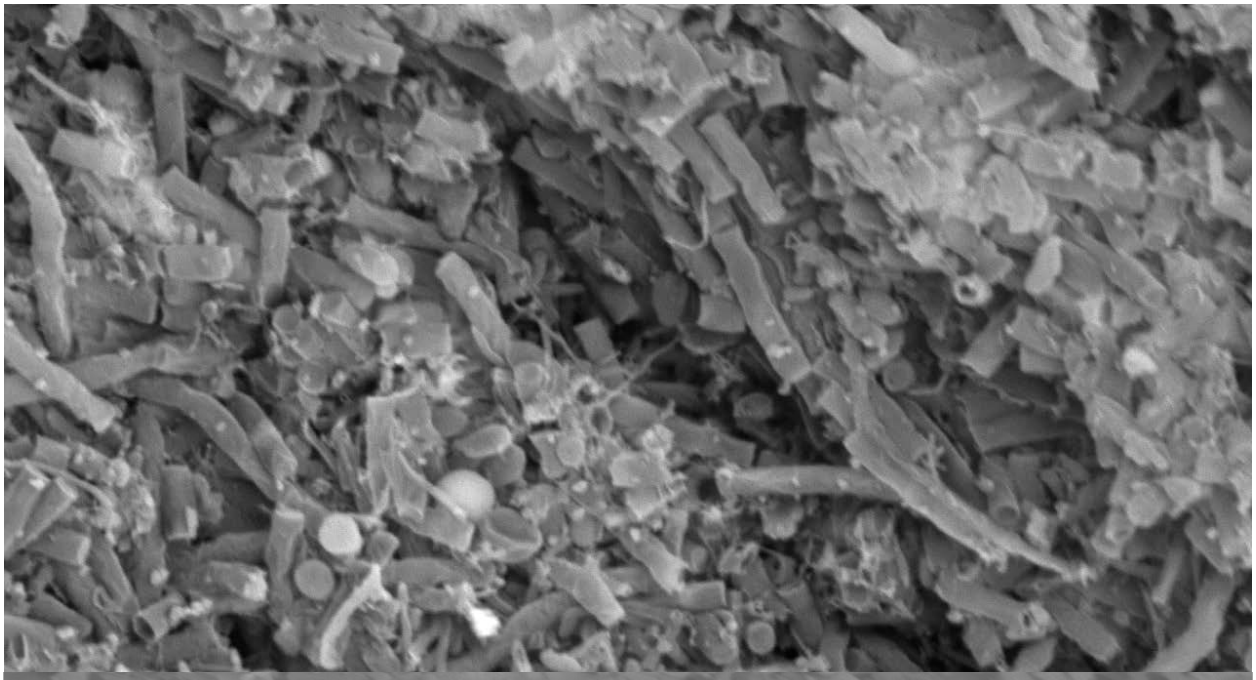

TRATAMIENTO BIOLÓGICO DE AGUAS RESIDUALES INDUSTRIALES MEDIANTE REACTORES ANAEROBIOS DE ALTA EFICACIA

BIOLOGICAL TREATMENT OF INDUSTRIAL WASTEWATER BY MEANS OF HIGH EFFICIENCY ANAEROBIC REACTORS

Doctoral dissertation, Madrid 2016
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Tratamiento biológico de aguas residuales industriales mediante reactores anaerobios de alta eficacia

Biological treatment of industrial wastewater by means of high efficiency anaerobic reactors

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D. Ángel Fernández Mohedano, Profesor Titular de Universidad profesor de la Sección Departamental de Ingeniería Química, perteneciente al Departamento de Química-Física Aplicada de la Universidad Autónoma de Madrid, y D. Víctor Monsalvo García, del Departamento de Innovación y Tecnología de FCC Aqualia

HACEN CONSTAR: que el presente trabajo, titulado: "Tratamiento biológico de aguas residuales industriales mediante reactores anaerobios de alta eficacia", presentado por Dña. Nuria García-Mancha Delgado-Ureña, ha sido realizado bajo su dirección, en los laboratorios de la Sección de Ingeniería Química, en la Universidad Autónoma de Madrid y que, a su juicio, reúne los requisitos de originalidad y rigor científico necesarios para ser presentado como Tesis Doctoral.

Y para que conste a efectos oportunos, firmamos el presente informe en Madrid, a 13 de septiembre de dos mil dieciséis.

Ángel Fernández Mohedano

Víctor Monsalvo García

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RESUMEN

La expansión de la actividad industrial lleva asociada un aumento de la cantidad de residuos generados, lo que ha conducido a la implantación de una estricta legislación ambiental, que regule la calidad de los principales efluentes generados para su vertido. El agua es un recurso cada vez más escaso, por lo que el tratamiento y la reutilización de las aguas industriales es un objetivo necesario para su mejor aprovechamiento como recurso natural. Una de las mayores amenazas de la calidad del agua es la contaminación química debida a la presencia de metales pesados, disolventes, pesticidas, etc. Estos compuestos químicos entran en el medio acuático causando toxicidad aguda y crónica para los organismos acuáticos, acumulación en el ecosistema, pérdida de hábitats y biodiversidad, así como efectos nocivos sobre la salud humana.

Ante esta situación general, la Directiva 2013/39/UE, recoge una lista de compuestos prioritarios que se caracterizan por ser tóxicos y/o persistentes, entre los que se incluyen hidrocarburos aromáticos policíclicos y herbicidas. El primer grupo de compuestos se han encontrado en concentraciones significativas en las aguas de lavado del proceso de recuperación de aceites usados, junto con un elevado grupo de hidrocarburos constituidos principalmente por derivados bencénicos y tolueno. Por otro lado, los plaguicidas son compuestos empleados habitualmente en diferentes cultivos. Las principales causas por las que estos compuestos aparecen en las aguas son por su empleo como práctica rutinaria en la agricultura, lavado de contenedores y equipos de dosificación, así como en aguas procedentes del lavado de frutas y hortalizas. Además de los compuestos incluidos en la lista, existen otros de especial interés ambiental debido a la toxicidad de los mismos, como es el caso de la morfolina. La morfolina es una amina secundaria que puede dar lugar a N-nitrosaminas (compuestos potencialmente mutagénicos y cancerígenos). Este compuesto y otros

inhibidores de corrosión se pueden encontrar en aguas residuales procedentes de la limpieza química de instalaciones industriales.

El avance experimentado por la tecnología anaerobia hace que se perfile como una alternativa para el tratamiento de aguas residuales económica y medioambientalmente atractiva debido a la baja producción de lodos, capacidad para tratar cargas orgánicas elevadas, producción energética y tolerancia a ciertos compuestos tóxicos. En concreto, los reactores anaerobios granulares de lecho expandido (EGSB, Expanded Granular Sludge Bed) ofrecen un gran potencial por su capacidad para tratar aguas con elevada carga orgánica y caudales elevados, aguas de baja carga, incluso aguas ácidas de baja carga en condiciones psicrofílicas (4-10 ºC), así como aguas residuales que contienen compuestos tóxicos y/o inhibitorios.

En la presente Tesis Doctoral se estudia la posibilidad de degradar mediante digestión anaerobia efluentes sintéticos que contienen inhibidores de corrosión (benzotriazol, quinoleína, morfolina y piperazina), así como herbicidas e insecticidas (ácido 2-metil-4-clorofenoxiacético (MCPA), imidacloprid y dimetoato). También se ha analizado el tratamiento mediante esta tecnología de efluentes industriales procedentes del lavado de tanques de fabricación productos fitosanitarios y del reciclado de aceites industriales usados.

El Capítulo III analiza la biodegradación anaerobia de diferentes inhibidores de corrosión como benzotriazol, quinoleína, morfolina y piperazina. En primer lugar se estudió la biodegradabilidad anaerobia en experimentos en discontinuo utilizando lodo granular no adaptado. Del mismo modo se evaluó la biodegradabilidad de diferentes mezclas (binarias, ternarias y cuaternarias) de los inhibidores de corrosión para establecer si se producen efectos sinérgicos o antagónicos por la presencia de estos compuestos simultáneamente. Los ensayos de biodegradabilidad revelaron que el benzotriazol no era degradado bajo condiciones anaerobias. Sin embargo, el resto de compuestos eran parcialmente biodegradados. En cuanto a los ensayos de biodegradabilidad de mezclas se observó que la degradación de

benzotriazol no se vio afectada por la presencia de otros inhibidores de corrosión, mientras la degradación de morfolina era ligeramente modificada. No obstante, la degradación de piperazina mejoró significativamente.

A continuación, se evaluó la inhibición que estos compuestos pueden provocar sobre la metanogénesis, observándose que la quinoleína era toxica para las arqueas acetoclásticas. Sin embargo, la metanogénesis hidrogenotrófica no se vio afectada por la presencia de inhibidores de corrosión.

Una vez estudiada la biodegradabilidad y toxicidad de estos compuestos sobre un fango anaerobio se procedió a tratar un agua sintética conteniendo estos compuestos en un reactor anaerobio de alta eficacia, tipo EGSB. La puesta en marcha del reactor EGSB se realizó utilizando una alimentación compuesta por glucosa, butirato, propionato y acetato hasta alcanzar valores constantes de eliminación de DQO y de producción de metano. Alcanzada la estabilidad del reactor, se incorporaron los inhibidores de corrosión a la alimentación del mismo, lo que redujo drásticamente la eliminación de DQO (demanda química de oxígeno) y la producción de metano. Sin embargo, después de 122 d de operación el sistema alcanzó una eficacia de eliminación de DQO (80 %) y de producción de metano (0,38 gCH_4-DQO/gDQO consumida) estable, consiguiendo la completa eliminación de quinoleína y piperazina. Los resultados obtenidos de la caracterización filogenética pusieron de manifiesto un predominio de genero *Methanosaeta* y del filo *Proteobacteria* al final del tratamiento.

En el Capítulo IV se realizó una primera aproximación sobre el tratamiento anaerobio de diferentes pesticidas. Para ello, se evaluó de forma individual la biodegradabilidad y la toxicidad de tres pesticidas comerciales cuyos ingredientes activos son MCPA (herbicida selectivo Fertiberia), imidacloprid (Couraze®) y Dimetoato (Danadim Progress®). Además, se llevó a cabo la puesta en marcha y la

aclimatación de un reactor EGSB para el tratamiento de esta mezcla de plaguicidas comerciales.

A partir de los ensayos de biodegradabilidad se puso de manifiesto que el MCPA es parcialmente biodegradable bajo las condiciones estudiadas. Sin embargo, el imidacloprid apenas fue degradado durante el tiempo que duro el ensayo (21 d). El dimetoato era completamente degradado cuando su concentración inicial era menor de 100 mg/L, concentración a partir de la cual se reduce la eliminación del mismo debido a la aparición de fenómenos de inhibición. También se evaluó la biodegradabilidad de ciclohexanona (disolvente mayoritario en la formulación Danadim Progress®), la cual era completamente degradada en el intervalo de concentraciones estudiado (10–500 mg/L). Como la composición de los pesticidas comerciales no era conocida, el estudio de biodegradabilidad también se analizó en términos de DQO y carbono orgánico total (COT). La reducción de DQO y COT en el caso del MCPA y del dimetoato, correspondía a la eliminación de los disolventes que contiene la formulación comercial y a la del propio herbicida, mientras que en el caso del imidacloprid era debida únicamente a la eliminación de los componentes que constituyen el Couraze®.

Los ensayos de inhibición de la metanogénesis acetoclástica mostraron una concentración inhibitoria máxima media (IC_{50}) de 474 y 367 mg/L para imidacloprid y dimetoato, respectivamente. Por otro lado, MCPA y ciclohexanona, no resultaron inhibitorios para las arqueas acetoclásticas.

Seguidamente se estudió el tratamiento de estos compuestos en un reactor anaerobio, tipo EGSB. La incorporación de los plaguicidas a la alimentación del reactor originó una drástica caída en la eficacia de eliminación de DQO y producción de metano. Después de 30 d de operación, el reactor se recuperó alcanzando eficacias de eliminación de DQO en torno al 85 % y una producción de metano de 0,9 gCH_4-DQO/gDQO consumida, pudiéndose incluso eliminar en su totalidad los insecticidas estudiados.

En el Capítulo V se aborda un estudio exhaustivo de la eliminación biológica de pesticidas comerciales en condiciones anaerobias. En primer lugar, se realizan ensayos de biodegradabilidad de 45 d de forma individual para cada pesticida, en los que se analizan los posibles intermedios con la finalidad de poder establecer las rutas de degradación. Del mismo modo, se realizó la biodegradabilidad de mezclas binarias y ternarias de los pesticidas para poder evaluar los posibles efectos sinérgicos o antagónicos. Los resultados de biodegradabilidad de cada pesticida muestran que el MCPA es degradado muy lentamente, de acuerdo con la baja eficacia de eliminación conseguida (20 %). El imidacloprid se degrada a través de la reducción del grupo nitro siguiendo un modelo en dos fases, una primera etapa donde la degradación es rápida, seguida de otra etapa de degradación lenta. El dimetoato se eliminó para todas las concentraciones estudiadas. La eliminación del dimetoato pudo iniciarse por el ataque al grupo alcoxi o por la eliminación del grupo metilo unido a la amina. También se estudió la biodegradabilidad de la ciclohexanona, obteniéndose que esta era transformada bajo condiciones anaerobias en su correspondiente alcohol (ciclohexanol). En los ensayos de biodegradabilidad de mezclas binarias y ternarias se observó que el MCPA no era degradado en presencia de otros compuestos, mientras que la degradación de imidacloprid mejoró (efecto sinérgico), aunque resultó necesaria una fase de aclimatación. Sin embargo, la eliminación de dimetoato se vio fuertemente afectada por la presencia de otros plaguicidas (efecto antagónico).

A continuación se evaluó el efecto inhibitorio que estos compuestos pueden tener sobre la actividad metanogénica del lodo (acetoclástica e hidrogenotrófica), así como la recuperación de la actividad una vez retirados los pesticidas del medio, pudiendo establecer si la inhibición es reversible (recuperación de la actividad) o irreversible (no se recuperó la actividad). El imidacloprid y el dimetoato causaron una inhibición irreversible sobre las arqueas acetoclásticas. Sin embargo, las aqueas hidrogenotróficas solo eran inhibidas irreversiblemente por el dimetoato. El MCPA y la ciclohexanona no causaron ningún efecto

inhibitorio sobre las arqueas acetoclásticas, llegando incluso a estimular a las arqueas hidrogenotróficas. La actividad de estas últimas también aumenta en presencia de imidacloprid.

El agua residual sintética, además de los plaguicidas comerciales, contenía peptona, extracto de levadura, leche en polvo, aceite de oliva, acetato de sodio, almidón y urea como fuentes de carbono. Tras la puesta en marcha del reactor y la correspondiente adaptación a la mezcla de pesticidas se aumentó la carga de los mismos, consiguiéndose tratar cargas de 87, 29 y 38 mg/L·d de MCPA, imidacloprid y dimetoato.

Al final del tratamiento se llevó a cabo la identificación de la biomasa mediante técnicas de biología molecular y el estudio de la morfología de los gránulos mediante microscopía electrónica de barrido (SEM, Scanning Electron Microscope). Los resultados mostraron el predominio de las arqueas hidrogenotróficas durante el tratamiento en el reactor, y las imágenes de SEM revelaron que a lo largo del tratamiento el granulo se hacía menos compacto, aumentando el número de agujeros y grietas.

En el Capítulo VI se estudió la viabilidad del tratamiento anaerobio de un agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios. El análisis de la composición del agua residual mostró una amplia heterogeneidad, con un elevado contenido en pesticidas, entre ellos varios herbicidas, insecticidas y fungicidas, así como una gran cantidad de disolventes orgánicos. Los ensayos de biodegradabilidad revelaron que al aumentar la carga contaminante de estas aguas se reducía la eliminación de DQO, poniendo de manifiesto la existencia de un fenómeno inhibitorio o que la etapa de hidrólisis/acetogénesis era muy lenta debido a la complejidad de las aguas objeto de estudio.

El estudio de inhibición sobre la metanogénesis mostro un comportamiento muy diferente entre las arqueas acetoclásticas e hidrogenotróficas, resultando fuertemente inhibidas las primeras, mientras que las últimas solo mostraron un 50 % de inhibición para la

concentración de DQO (12,8 gDQO/L) más alta empleada en el ensayo.

El tratamiento del agua residual industrial se realizó en un reactor EGSB que operó en primer lugar bajo condiciones mesofílicas (35 ºC), obteniéndose una eficacia de eliminación de DQO del 33 %. Con el objetivo de aumentar la mineralización de la materia orgánica y, por lo tanto, la producción de metano se elevó la temperatura del reactor a 55 ºC (condiciones termofílicas). Con el aumento de temperatura se mejoró la eficacia de eliminación de DQO y la de producción de metano, y se observó un gran cambio en las poblaciones microbianas presentes en el lodo granular. Sin embargo, la eliminación de los plaguicidas que contenía el agua residual industrial mostró similares resultados para las dos temperaturas ensayadas.

Finalmente, se evaluó la posibilidad de acoplar un post-tratamiento aerobio para aumentar aún más la eliminación de DQO. Para ello se realizó, mediante técnicas respirométricas, un ensayo de biodegradabilidad aerobia de los efluentes obtenidos del tratamiento anaerobio a 35 ºC y a 55 ºC. El análisis de la biodegradabilidad aerobia reveló que ambos tratamientos biológicos (anaerobio y aerobio) podrían ser acoplados, consiguiendo una eficacia de eliminación total de DQO del 62 %.

El último capítulo (Capítulo VII) se centra en el estudio del tratamiento biológico de un agua residual industrial procedente del reciclado de aceites industriales usados mediante un reactor anaerobio de alta eficacia. Al igual que en los capítulos anteriores, se llevaron a cabo ensayos para determinar la biodegradabilidad del agua residual y los posibles efectos inhibitorios que esta puede tener sobre la metanogénesis. Los ensayos de biodegradabilidad mostraron que agua residual industrial era parcialmente biodegradable en condiciones mesofílicas. En cuanto a los ensayos de estimulación/inhibición de la metanogénesis, se observó que el empleo de bajas cargas del agua residual aumentaban tanto la actividad metanogénica especifica acetoclástica como la hidrogenotrófica.

El agua residual se sometió a un tratamiento anaerobio empleando un reactor EGSB que operó a temperatura ambiente (17–21 ºC) durante los primeros 67 d. Durante ese periodo, al aumentar la velocidad de carga orgánica (OLR, Organic Loading Rate) se observó una leve disminución de la eficacia del proceso. Sin embargo, un aumento de la OLR hasta 10 gDQO/L·d provocó una drástica disminución de la eficacia de eliminación de DQO y producción de metano, así como la aparición de una elevada concentración de acetato en el efluente del reactor. Para recuperar la eficacia del reactor se aumentó la temperatura a 32 ºC, consiguiéndose una eficacia de eliminación de DQO y de producción de metano del 75 y 30 %, respectivamente. El análisis de la composición del agua residual objeto de estudio mostró una amplia heterogeneidad (alcoholes, compuestos fenólicos, alcanos lineales y alcanos cíclicos), identificándose como componente mayoritario el etilenglicol, el cual no fue detectado en el efluente obtenido después del tratamiento. Por otro lado, se llevó a cabo la identificación de la biomasa, antes y después del tratamiento, observándose cambios significativos en la composición microbiana del lodo granular. Al final del mismo, se detectaron arqueas relacionadas con el tratamiento de aguas residuales que contienen alcanos, poniendo de manifiesto la especialización de la biomasa a lo largo del tratamiento.

SUMMARY

The intensification of industrial activities has caused an increase of wastes production, which has led to the establishment of strict environmental regulations to standardize the quality of the effluents to be discharged. Thus, the treatment and reuse of industrial wastewaters is necessary to reduce water consumption and the environmental impact on water reservoirs. Chemical contamination is one of the greatest threats regarding water sources conservation, mainly the presence of heavy metals, solvents, pesticides, etc. These compounds can enter into the aquatic environment causing acute and chronic toxicity to aquatic organisms, accumulation in the ecosystem and losses of habitats and biodiversity, as well as harmful effects on human health.

Strict regulations have stablished maximum acceptable concentrations of these compounds to control their presence in wastewater before discharge. The Directive 2013/39/UE contains a list of priority pollutants which are toxic and/or persistent, including several polycyclic aromatic hydrocarbons and pesticides. These compounds have been found at high relative concentrations in wastewaters generated in the recovery process of used industrial oil, which contain some hydrocarbons such a benzene derivate and toluene. Pesticides are worldwide used as a routine practice in agriculture, which are commonly found in surface runoffs. The cleaning of recipient and dispensing equipments, the preparation of agricultural products such as fruit and vegetable washing, and disposal of polluted plants are other important sources of high-concentrated pesticides bearing wastewaters. Furthermore, corrosion inhibitors are found in wastewater resulted from chemical cleaning of industrial facilities. Owing to their toxicity and low-biodegradability, these compounds are of special environmental and health interest like morpholine (potential

mutagenic and carcinogenic agent), which are not included in this EU priority pollutants list.

High-rate anaerobic technologies have experienced a remarkable improvement in the last decades making them an economically and environmentally attractive alternative for wastewater treatment due to the low production of excess biosolids, is suited for the treatment of high strength wastewaters, small foot-print, useful energy is produced (biogas) and tolerates several toxic compounds. Specially, expanded granular sludge bed (EGSB) anaerobic reactors offers a great potential to treat high organic and hydraulic loadings, medium strength wastewaters, even under psychrophilic conditions (4-10 ºC), as well as wastewater containing hardly biodegradable and/or toxic compounds.

In this work, the feasibility of anaerobic systems for the abatement of corrosion inhibitors (benzotriazole, quinoline, morpholine and piperazine), wastewaters polluted with herbicides (2-methyl-4-chlorophenoxyacetic acid, MCPA) and insecticides (imidacloprid and dimethoate) has been studied. In addition, two industrial wastewaters collected from a pesticides factory and from used industrial oils recovery have been treated in a high-rate anaerobic (EGSB) reactor.

In Chapter III, the anaerobic biodegradability of different corrosion inhibitors (benzotriazole, quinoline, morpholine and piperazine) is discussed. These assays have been conducted individually (single-compound spiking solutions) and in form of different mixtures (binary, tertiary and quaternary spiking solutions) of the selected corrosion inhibitors using non-adapted granular sludge. Interactions in the biodegradability (synergistic and antagonistic effects) of the target compounds have been identified by comparing the results from experiments carried out individually and in mixtures. The results from these experiments have revealed that benzotriazole is a recalcitrant species under anaerobic conditions, while the rest of corrosion inhibitors were partially degraded. The biodegradability of benzotriazole was not affected by the presence of other corrosion inhibitors, morpholine biodegradation was slightly modified, quinoline

removal was severely affected and rapid biodegradability of piperazine was significantly improved.

In addition, the effect of these compounds over the methanogenesis activity was evaluated, showing that acetoclastic archaea was affected by the toxic effect of quinoline. However, hydrogenotrophic methanogenesis remained unaltered by the presence of corrosion inhibitors.

Once the biodegradability and toxicity of these compounds over anaerobic sludge had been studied the treatment of a synthetic wastewater containing these compounds was carried out by using a high-rate reactor (EGSB). The start-up of the EGSB reactor was performed using a feed containing glucose, butyrate, propionate and acetate until reaching a constant chemical oxygen demand (COD) removal efficiency and methane production. Then, corrosion inhibitors were added into the feed, causing a drastically drop in COD removal and methane production. Nevertheless, after 122 d the system achieved a stable COD removal efficiency (80 %) and methane production (0.38 gCH_4-COD/gCOD consumed). The phylogenetic characterization revealed the prevalence of genus *Methanosaeta* and the phylum *Proteobacteria* at the end of the treatment.

In the Chapter IV a preliminary study about the treatment of pesticides is presented. For this purpose, the anaerobic biodegradability and toxicity of each pesticide whose active agents are MCPA (selective herbicide Fertiberia), imidacloprid (Couraze®) and Dimetoato (Danadim Progress®) have been assessed. The start-up and acclimation of an EGSB reactor was satisfactorily performed for treating wastewaters polluted with pesticides.

Biodegradability assays showed that MCPA was partially biodegraded under the operation conditions studied. However, imidacloprid was not removed during the assay (21 d). Dimethoate was completely degraded when the starting concentration was lower than 100 mg/L, since higher concentrations decreased the removal efficiency due to

inhibition phenomena. In addition, the biodegradability of cyclohexanone (the main solvent in Danadim Progress® formulation) has been evaluated, which was completely removed within the concentration range studied (10–500 mg/L). The composition of the commercial pesticides was not known, thus, the biodegradability was analyzed in terms of organic matter parameters: COD and total organic carbon (TOC). COD and TOC reduction observed in MCPA and dimethoate biodegradability tests was associated to the removal of solvents contained in the commercial formulation and the pesticide itself. In the case of imidacloprid, the organic matter removal was exclusively due to the consumption of solvents present in Couraze®.

Subsequently the treatment of these compounds in an anaerobic reactor (EGSB) was carried out. COD removal efficiency and methane production drastically decreased when pesticides were spiked in the reactor feed. After 30 d of acclimation period, the reactor recovered the COD removal (85 %) and the methane production (0.9 gCH_4-COD/gCOD consumed) efficiencies. The degradation of the insecticides improved along the experiment, and a free-insectides effluent was achieved.

In the Chapter V, the biological degradation of commercial pesticides under anaerobic conditions is exhaustively discussed. First, biodegradability of pesticides was studied during 45 d individually. Intermediates were analyzed in order to propose the degradation pathways. Similarly, the biodegradability of binary and tertiary mixtures of pesticides has been studied to evaluate the synergistic/antagonistic effects. The results from the individual biodegradability analysis showed that MCPA is slowly degraded, achieving a low removal efficiency of 20 %. Imidacloprid was degraded by the reductive reaction of nitro group, reaching high initial degradation rates following a biphasic model. Dimethoate was removed within the concentration range studied, whose degradation initiated by the hydrolytic attack at the alcoxi group or by the demethylation of the methylamine moiety of dimethoate. Cyclohexanone was effectively transformed into cyclohexanol under

anaerobic conditions. Biodegradability assays of binary and tertiary mixtures revealed that MCPA was not degraded in presence of other pesticides, while imidacloprid degradation was improved (synergistic effect) after an acclimation phase. However, dimethoate degradation was strongly affected (antagonistic effects) by the presence of other pesticides.

The inhibitory effects caused by phytosanitary compounds over the granular sludge methanogenic activity, as well as the impact and type of inhibition (reversible or irreversible) have been assessed. Imidacloprid and dimethoate caused an irreversible inhibition over the acetoclastic archaeas. However, hydrogenotrophic archaeas were more robust against the presence of pesticides, whose activity was only irreversibly inhibited by dimethoate. MCPA and cyclohexanone did not cause any inhibitory effect over acetoclastic biomass, which even stimulated hydrogenotrophic microorganisms. These last microorganisms also enhanced their activity in presence of imidacloprid.

Afterward, the treatment of a synthetic wastewater containing commertial pesticides was carried out by means an anaerobic reactor (EGSB). In addition to pesticides, the synthetic wastewater was supplemented with peptone, yeast extract, milk powder, sunflower oil, sodium acetate, starch and urea as carbon sources. After the start-up and the acclimation phase the EGSB reactor was fed with a mixture of pesticides, the pesticides loading rate was gradually increased at 87, 29 y 38 mg/L·d of MCPA, imidacloprid y dimethoate.

At the end of the long-term experiment the resulting biomass was characterized by molecular biological techniques and scanning electron microscopy (SEM). The results revealed that hidrogenotrophic biomass prevailed along the treatment and the SEM images showed a less compact granule in which the number of cracks and holes increased, which suggest a partial disaggregation of the granules.

In the Chapter VI industrial wastewater collected from a pesticides factory were treated by anaerobes. This wastewater is characterized by a heterogeneous composition with a high concentration of a complex mixture of pesticides, including herbicides, insecticides, fungicides and organics solvents. Biodegradability assays showed a decrease in the COD removal efficiency at increasing the COD fraction associated to the wastewater. This fact can be caused by the occurrence of inhibitory phenomena or a limiting hydrolysis/acetogenesis rate associated to the complex composition of the wastewater studied.

The study of the inhibitory effect over methanogenesis revealed a different behavior of acetoclastic and hydrogenotrophic archaea. The formers were strongly inhibited, but the activity of the hydrogenotrophic biomass was partially reduced by a 50 % when treating a highly concentrated wastewater (12.8 gCOD/L).

The wastewater treatment in an EGSB reactor at mesophilic conditions (35 ºC) led to a COD removal efficiency of 33 %. In order to improve the mineralization rate and the methane production, temperature was increased to 55 ºC (thermophilic conditions), which enhanced the COD removal and methane production. In parallel, the microbial population significantly changed but, pesticide removal efficiency remained constant. For this reason, the possibility of an aerobic post-treatment was evaluated for the removal of the organic matter present in the resulting effluents from the EGSB reactor. For this purpose, respirometric analyses were carried out to study the aerobic biodegradability of the effluents from the EGSB when operated at mesophilic and thermophilic conditions. The results revealed that both systems (anaerobic and aerobic) could be combined, achieving a COD removal efficiency of 62 %.

Last chapter (Chapter VII) focuses on the study of anaerobic treatment of wastewater from used industrial oil recovery in an EGSB reactor. The biodegradability and stimulation/inhibition assays showed that this wastewater can be partially degraded at mesophilic conditions. Methanogenic stimulation/inhibition assays revealed that

low-strength wastewater (≤ 2 gCOD/L) increased both acetoclastic and hidrogenotrophic activity of the granular biomass inoculated. An EGSB reactor was operated at room temperature (17–21 ºC) during 67 d treating wastewater from an industrial oil recovery plant. The organic loading rate (OLR) was gradually increased along the experiment, which caused a slight reduction of the COD removal efficiencies. However, the increase of the OLR up to 10 gCOD/L·d caused a significant drop of the COD removal and methanogenic efficiency, and also a high acetate concentration was detected in the effluent. In order to recover the reactor efficiency, the temperature was increased to 32 ºC, achieving a COD removal and methane production of 75 and 30 %, respectively. Most of the relevant compounds present in the feed (alcohols, phenolic compounds, linear alkanes and cyclic alkanes) were not detected in the resulting effluent. In addition, the biomass identification along the treatment revealed the presence of archaeas related to the treatment of alkanes bearing wastewater, highlighting the specialization of the biomass.

1

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. LA PROBLEMÁTICA DEL AGUA

En la superficie de la Tierra hay aproximadamente 1.400 millones de km^3 de agua, de los cuales solo 35 millones son agua dulce. Sin embargo, no toda esa cantidad de agua dulce se encuentra accesible. Las Naciones unidas estiman que solo 200.000 km^3 de agua dulce estarían disponibles para consumo humano, poniendo de manifiesto que el agua es un recurso escaso. Además, su distribución no es equitativa, cerca de 1.200 millones de personas viven en áreas donde el agua escasea físicamente (Water, U. N., 2006, y FAO, 2007).

El continuo crecimiento de la población, el aumento del nivel de vida, el cambio climático, la industrialización, la agricultura y la urbanización agravan el problema de escasez y disponibilidad (Singh, 2007).

Según Naciones Unidas, el sector doméstico solo representa el 10 % del uso total de agua mientras que la agricultura es, con diferencia, el mayor consumidor de agua a nivel mundial, representando el 70 % de las extracciones de agua en todo el mundo, aunque esta cifra varía considerablemente entre países. La industria y la energía juntas representan el 20 % de la demanda de agua. Los países más desarrollados tienen una proporción mucho mayor de extracciones de agua dulce para la industria que los países menos desarrollados, donde predomina la agricultura (www.un.org).

El sector productor no sólo es el que más gasta, también es el que más contamina (Tabla 1.1). Según el Programa de ONU-Agua para la Promoción y la Comunicación en el marco del Decenio más del 80 % de las aguas residuales generadas en los países en desarrollo se descargan sin tratamiento a cuerpos de agua superficiales. A nivel mundial, 2 millones de toneladas de aguas residuales, desechos industriales y agrícolas se vierten en las aguas del mundo.

Tabla 1.1. Fuentes de contaminación del agua dulce, efectos y principales constituyentes (Water, U. N., 2006)

Tipo de contaminación	Fuente primaria	Efectos[1]	Principales constituyentes[2]
1. Materia orgánica	Vertido de residuos industriales y domésticos.	Falta de oxígeno en la columna de agua, a medida que ésta se descompone sufre estrés o ahoga la vida acuática.	Demanda Biológica de Oxígeno (DBO), Carbono Orgánico Disuelto (COD) y Oxígeno Disuelto (OD).
2. Patógenos y contaminantes microbianos	Residuos domésticos, ganado y otros animales de granja, fuentes naturales.	Propaga enfermedades infecciosas a través de la red de abastecimiento de agua potable, provocando la aparición de enfermedades diarreicas y de parásitos intestinales, alta tasa de mortalidad infantil en los países en vías de desarrollo.	*Shigella*, *Salmonella*, *Criptosporidium*, y Escherichia coli.
3. Nutrientes	Principalmente debido a la escorrentía en tierras agrícolas y áreas urbanas, pero también a causa de los vertidos industriales.	Sobreestimula el crecimiento de algas (eutrofización), que posteriormente se descomponen, privando de oxígeno al agua y dañando la vida acuática. Los altos niveles nitratos en el agua potable provocan de enfermedades en la población.	Nitrógeno total (orgánico e inorgánico), fósforo total (orgánico e inorgánico). En caso de eutrofización: Oxígeno Disuelto, especies individuales de N (NH_4^+, NO_2^-, NO_3^-, N Orgánico, Ortofosfato).

4. Salinización	Lixiviación de suelos alcalinos por exceso de irrigación o de bombeo de los acuíferos costeros, que resulta en una intrusión de agua salada.	La acumulación de sal en el suelo, acaba con los cultivos y reduce las cosechas. El agua deja de ser potable.	Conductividad eléctrica, cloruro, seguido de una caracterización de cationes principales (Ca^{2+} y Mg^{2+}) y aniones.
5. Acidificación (precipitación y escorrentía)	Sulfuro, óxidos de nitrógeno y partículas procedentes de la producción de energía eléctrica, la masificación industrial y las emisiones de vehículos y camiones (deposiciones húmedas y secas). Residuos procedentes de drenaje de las minas con ácidos y de las propias minas.	Incrementa la acidificación de lagos y arroyos, lo cual afecta negativamente a los organismos acuáticos y provoca la lixiviación de metales pesados como el aluminio en las masas de agua.	pH.
6. Metales pesados	Industrias y minas.	Subsiste en medios de agua dulce, como en sedimentos fluviales y humedales durante largos periodos. Se acumula en los tejidos de los peces y el marisco. Resulta tóxico para todo organismo humano o acuático que lo consuma.	Pb, Cd, Zn, Cu, Ni, Cr, Hg, As (en especial en las aguas subterráneas).
7. Componentes orgánicos tóxicos y microorganismos contaminantes[3]	Gran variedad de orígenes: terrenos industriales, automóviles, granjeros, jardineros, vertederos municipales.	Una amplia gama de efectos tóxicos en la fauna acuática y también en los humanos, que van desde una ligera inmunodepresión hasta un envenenamiento grave o la incapacidad de procrear.	PAH, PCB, pesticidas (lindano, DDT, PCP, aldrin, dieldrin, isodrin, hexaclorobenceno).

8. Térmica	La fragmentación de los ríos a causa de la construcción de presas y depósitos que ralentizan el curso del agua y hacen que ésta se caliente. Industrias con torres de refrigeración y otras descargas de temperatura por encima de la temperatura ambiente por medio de conductos.	Cambio en los niveles de oxígeno y en la tasa de descomposición de la materia orgánica en la columna de agua. Puede alterar la composición de especies en la masa de agua receptora.	Temperatura.
9. Partículas de tierra en suspensión	La erosión natural del suelo, la agricultura, la construcción de carreteras y otros cambios en los usos de la tierra.	Reduce la cantidad de agua potable y la de recreo, degrada de los hábitats acuáticos llenándolos de partículas de arcilla, interrumpe las puestas de huevos e interfiere en la alimentación.	Sólidos en suspensión totales y turbidez.

Otros contaminantes incluyen la radioactividad, el flúor o el selenio.
1 Principalmente de Revenga y Mock (2000). Su recopilación de Taylor y Smith (1997); Shiklomanov (1997); PNUMA/GEMS (1995).
2 De N.E. Peters, B. Webb. Comunicación personal (2004).
3 La lista de microorganismos contaminantes incluye actualmente una serie de perturbadores endocrinos, antioxidantes, plastificadores, retardadores del fuego, repelentes de insectos, disolventes, insecticidas, herbicidas, fragancias, aditivos alimentarios, medicamentos de prescripción médica y productos farmacéuticos (anticonceptivos, antibióticos, etc.), productos sin prescripción médica (cafeína, nicotina y derivados y estimulantes).

1.2. AGUAS RESIDUALES INDUSTRIALES

En la Directiva 91/271 CEE, del Tratamiento de Aguas Residuales Urbanas, se definen los distintos tipos de aguas residuales:

- Aguas residuales domésticas: procedentes de viviendas y de servicios, generadas principalmente por el metabolismo humano y las actividades domésticas.
- Aguas residuales industriales: aguas residuales vertidas desde locales utilizados para efectuar cualquier actividad comercial o industrial, excluyendo las aguas residuales domésticas y las de escorrentía pluvial.
- Aguas urbanas: aguas residuales domésticas o la mezcla de las mismas con aguas residuales industriales y/o aguas de escorrentía pluvial.

La composición de las aguas residuales urbanas no varía sustancialmente de unas poblaciones a otras, mientras que las aguas residuales industriales presentan características muy diversas, variando el caudal y la composición en función de la industria que las genera (Tabla 1.2). Debido a la elevada variabilidad que presentan es difícil establecer una clasificación de los efluentes generados, aunque una posibilidad es realizarla en función de su biodegradabilidad y toxicidad. Siguiendo este criterio, los efluentes industriales se podrían dividir en aguas residuales industriales con:

- Alta demanda biológica de oxígeno (DBO) y nula o muy baja concentración de agentes tóxicos, como los procedentes de las industrias agroalimentarias.
- Alta DBO y toxicidad, como las de fabricación de pastas celulósicas, refinerías de petróleo, alpechines, vinazas, entre otros.

Tabla 1.2. Características típicas de aguas residuales industriales (MA: muy alta, A: alta, M: media, B: baja) (Gray, 2010).

Industria	Caudal	DBO	SST	DQO	pH	Nitrógeno	Fósforo
Cárnica	Intermitente	A–MA	A	A-MA	Neutro	Presente	Presente
Lechera	Intermitente	M-A	B-M	M-A	Ácido – alcalino	Adecuado	Presente
Quesera	Intermitente	MA	M-MA	MA	Ácido – alcalino	Deficiente	Presente
Bebidas alcohólicas	Intermitente	A-MA	B-A	A-MA	Alcalino	Deficiente	Deficiente
Bebidas	Intermitente	M-A	B-A	M-A	-	Deficiente	Presente
Textil	Intermitente - continuo	A	A	A	Alcalino	Deficiente	Presente
Curtido y acabado	Intermitente	MA	MA	MA	Ácido – alcalino	Adecuado	Deficiente
Galvanizado	Continuo - variable	B	M-A	B	Ácido	Presente	Presente
Frutas y verduras	Intermitente	M-MA	M-MA	-	Ácido – alcalino	Deficiente	Deficiente
Papelera	Continuo	M-MA	B-A	B-A	Neutro	Deficiente	Deficiente
Farmacéutica	Continuo – intermitente	A	B-A	A	Ácido – alcalino	Deficiente	Deficiente
Plásticos y resinas	Continuo - variable	M-A	B-A	M-A	Ácido – alcalino	-	-

- Baja DBO y alta toxicidad, típicas, de la industria química, farmacéutica y metalúrgica.

Cada una de ellas debe desarrollar y aplicar los métodos de tratamiento más adecuados para conseguir cumplir los requerimientos de vertido establecidos por la normativa vigente.

1.2.1. MARCO LEGAL

La protección de la calidad del agua de todas las fuentes de aguas residuales no tratadas, ya sean domésticas, industriales o agrícolas, es un prerrequisito para asegurar el desarrollo sostenible, la reducción de la pobreza, la salud humana y del ecosistema y el bienestar de las personas. La legislación ambiental relativa a la calidad del agua se basa en gran medida en las normas de calidad referentes a la idoneidad para un uso específico, la protección de las aguas receptoras o límites de emisión de vertidos.

La disposición de mayor relevancia de los últimos años ha sido la denominada Directiva Marco del Agua (Directiva 2000/60/CE), por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas. Esta Directiva persigue mejorar la protección de las aguas en sus aspectos cuantitativos y cualitativos, fomentar su uso sostenible y proteger los ecosistemas acuáticos, así como salvaguardar y desarrollar los usos potenciales de las aguas. Esta Directiva ha sido modificada varias veces por:

- La Decisión nº 2455/2001/CE, mediante la cual se aprobó la lista de sustancias prioritarias en el ámbito de la política de aguas entre las que se encuentran metales pesados y compuestos orgánicos tales como plaguicidas, hidrocarburos aromáticos policíclicos (PAHs) o surfactantes.
- La Directiva 2008/105/CE por la que se procede a la modificación y establecimiento de nuevas normas de calidad ambiental, se añade nuevas fórmulas y obligaciones de

coordinación; ahora se fija una revisión periódica del anexo X de la Directiva de Aguas. Así mismo, se establecen disposiciones específicas para determinadas sustancias; se introduce la figura de la denominada lista de observación de sustancias, sobre las que habrán de recabarse datos de seguimiento a nivel comunitario que sirvan de base para futuros ejercicios de asignación de prioridad; también se establecen disposiciones específicas para sustancias farmacéuticas, entre otras cuestiones.

- La Directiva 2009/90/CE, que establece las especificaciones técnicas del análisis químico y el seguimiento del estado de las aguas (recogidas en el Real Decreto 60/2011 sobre las normas de calidad ambiental en la política de aguas)

- La Directiva 2013/39/UE por la que se actualiza la lista de sustancias prioritarias mediante la identificación de nuevas sustancias para acciones prioritarias a escala de la Unión, y se adoptan medidas para lograr el buen estado químico de las aguas superficiales mediante la adopción de normas de calidad ambiental para las sustancias prioritarias y otros contaminantes determinados.

La Directiva de Control y Prevención Integrada de la Contaminación (96/61/CE), para el control de la contaminación con sustancias peligrosas procedentes de industrias y la reutilización de esas aguas mediante el desarrollo de tecnologías y prácticas de gestión para sectores industriales específicos, se traspone al ordenamiento jurídico español como la Ley 16/2002, de prevención y control integrados de la contaminación, y la puesta en marcha del PRTR-España (Registro Estatal de Emisiones y Fuentes Contaminantes), cuyo objetivo es reducir el vertido de algunos contaminantes específicos y emplear sistemas avanzados de tratamiento de aguas residuales in situ. Esta ley recoge una lista de las principales sustancias contaminantes para las que han de fijarse valores límite de emisiones a las aguas, teniendo en

cuenta las mejores técnicas disponibles, las características técnicas de la instalación y su localización geográfica:

- Compuestos órgano-halogenados y sustancias que puedan dar origen a compuestos de esta clase en el medio acuático.
- Compuestos organofosforados.
- Compuestos organoestánicos.
- Sustancias y preparados cuyas propiedades cancerígenas, mutágenas o que puedan afectar a la reproducción en el medio acuático.
- Hidrocarburos persistentes y sustancias orgánicas tóxicas persistentes y bioacumulables.
- Cianuros.
- Metales y sus compuestos.
- Arsénico y sus compuestos.
- Biocidas y productos fitosanitarios.
- Materias en suspensión.
- Sustancias que contribuyen a la eutrofización (en particular nitratos y fosfatos).
- Sustancias que ejercen una influencia desfavorable sobre el balance de oxígeno (y computables mediante parámetros tales como la DBO y DQO).

En materia de aguas residuales, la Directiva 91/271/CEE, relativa al tratamiento de las aguas residuales urbanas, señala la necesidad de que los vertidos industriales que entren en los sistemas colectores e instalaciones de tratamiento de aguas residuales urbanas se sometan a un tratamiento previo para garantizar que no provoquen efectos

nocivos sobre las personas y el medio ambiente. En este contexto, la Comunidad de Madrid aprobó la Ley 10/1993, que tiene por objeto regular los vertidos industriales al sistema integral de saneamiento, fijando límites de vertido de concentración de parámetros de tipo global (DBO_5, DQO y sólidos en suspensión), de determinados compuestos y de toxicidad biológica. En el artículo 4 de esta Ley se establece la necesidad de tratar las aguas residuales procedentes de vertidos industriales que no se ajusten a las características recogidas antes de ser incorporadas a la red de alcantarillado o incluso la obligación de disminuir los procesos de fabricación si el tratamiento no consigue bajar los niveles de sustancias contaminantes hasta los límites establecidos.

1.2.2. TRATAMIENTO DE AGUAS RESIDUALES INDUSTRIALES

Los sistemas de depuración de aguas residuales industriales tratan de devolver el agua al medio natural con unas características físicas, químicas y biológicas lo más parecidas a su estado natural. La heterogeneidad de las aguas residuales industriales se traduce en una gran diversidad de tratamientos para su depuración. La selección del método o combinación de métodos más adecuado se realiza teniendo en cuenta tanto las características específicas de los efluentes, como los requerimientos de depuración, sometidos, en todo caso, al análisis de coste resultante de las características técnicas, las disponibilidades de espacio, necesidades de personal, mantenimiento y condiciones de operación. La Tabla 1.3 recoge los métodos de tratamientos de aguas residuales industriales más habituales.

Tabla 1.3. Principales procesos unitarios en el tratamiento de aguas residuales (Gray, 2010).

PROCESO	DESCRIPCIÓN
Procesos físicos	
Homogeneización	Se emplea para conseguir un caudal de tratamiento constante cuando el caudal de agua residual producida varía con el tiempo. La homogenización es una práctica útil en plantas de tratamiento pequeñas que experimentan variaciones de caudal y carga contaminante.
Desbaste	Eliminación de partículas de gran tamaño de aguas residuales. Se usa como pretratamiento para evitar que dañen equipos en los tratamientos posteriores.
Sedimentación	Separación por gravedad de sólidos orgánicos e inorgánicos del agua residual.
Flotación	Eliminación de partículas en suspensión mediante generación de burbujas de aire, provocando que estas se asocien a las partículas presentes en el agua residual y sean arrastradas hacia la superficie y sacadas del sistema. Se utiliza principalmente en industrias lácteas, papeleras, cárnicas y de producción de pintura.
Hidrociclones	Eliminación de arena, grava y vidrio del agua residual mediante su introducción, de forma tangencial, en un tanque cónico. Las fuerzas centrífugas lanzan la arena sobre las paredes del cono, donde los sólidos van deslizándose hacia el punto de salida inferior. El agua residual libre ya de arena (contiene todavía solidos orgánicos) circula hacia el centro del vórtice y sale por la parte superior.

Filtración	Reducción de la concentración de sólidos en suspensión (< 20 mg/L). Se pueden llegar a eliminar partículas entre 1 y 5 μm empleando filtros de fibra sintética.
Centrifugación	Separación de sólidos de la corriente acuosa. Ampliamente utilizado en la industria farmacéutica, papelera, química y alimentaria, y en la deshidratación de lodos de depuradora.
Osmosis inversa	El agua residual es sometida a presión (1500–3000 kPa) y forzada atravesar una membrana semi-permeable con poros extremadamente pequeños para concentrar iones y otras partículas presentes en el agua residual. Se emplea para extraer los contaminantes del agua residual.
Ultrafiltración	Similar a la ósmosis inversa. Empleando presiones de hasta 3000 kPa, el agua es forzada a atravesar una membrana microporosa, eliminándose partículas de 0,005-0,1 μm. Se utiliza para la eliminación y reciclaje de material coloidal incluyendo tintes, aceites, pinturas e incluso proteínas del suero de la leche y del queso de las aguas residuales. Capaz de eliminar los microorganismos más pequeños, incluidos los virus.
Microfiltración	Similar a la ultrafiltración, pero para la eliminación de partículas de gran tamaño (0,1-5 μm) empleando presiones más bajas (100-400 kPa). Ampliamente utilizado en la industria alimentaria y de producción de bebidas. Los filtros microporosos también pueden ser utilizados para la desinfección de aguas.

Adsorción	El proceso de adsorción consiste en la captación de sustancias solubles en la superficie de un sólido empleando resinas sintéticas o carbón activado. Se utiliza principalmente para la eliminación de compuestos orgánicos.
Procesos químicos	
Neutralización	Adición de un ácido (por ejemplo, H_2SO_4) o una base (por ejemplo NaOH) al agua residual para ajustar su pH (neutro) para proteger los equipos de tratamiento. Ampliamente utilizado en la industria química y farmacéutica.
Precipitación	Eliminación de componentes inorgánicos disueltos por precipitación en forma de un sólido, mediante la adición de un ácido o una base, o cambiando la temperatura. El precipitado se puede eliminar por sedimentación, flotación u otros procesos.
Intercambio Iónico	Mediante el empleo de resinas de intercambio iónico los iones disueltos en el agua son retenidos selectivamente. Por ejemplo iones Ca^{2+} y Mg^{2+} pueden reemplazar a los iones Na^{+} de una resina reduciendo así la dureza del agua.
Oxidación-reducción	Los compuestos orgánicos e inorgánicos presentes en aguas residuales pueden ser eliminados, o su toxicidad puede reducirse, mediante la transferencia electrónica entre el reactivo y el contaminante.

Procesos biológicos	
Lodos activos	Consiste en poner en contacto el agua residual con microorganismos en un medio aereado para permitir que las bacterias degraden la materia orgánica contaminante (reducción de 95 %). La biomasa microbiana y efluente tratado se separan por sedimentación y una parte de la biomasa (lodos) se recircula al tanque de aereación para mantener la concentración de microorganismos.
Filtración biológica	El agua residual se distribuye sobre un lecho inerte sobre el que se desarrollan microorganismos y degradan la materia orgánica presente. La aereación se produce a través de ventilación natural.
Lagunas de estabilización	Grandes lagunas donde se almacena el agua residual durante largos períodos para permitir que los microorganismos degraden la materia orgánica. Existen diferentes tipos como lagunas aereadas, no aereadas y anaerobias. Algunos diseños se basan en las algas para proporcionar oxígeno para la descomposición bacteriana de la materia orgánica.
Digestión anaerobia	Se utiliza para efluentes con alta carga orgánica (por ejemplo, la industria farmacéutica, alimentaria y de producción de bebidas). El agua residual se almacena en un tanque sellado sin oxígeno. Las bacterias anaerobias descomponen la materia orgánica en metano, dióxido de carbono y ácidos grasos volátiles (AGVs). El efluente final todavía requiere un tratamiento adicional ya que tiene una alta DBO. También se utiliza para la estabilización de lodos de depuradora.

1.3. TRATAMIENTO ANAEROBIO DE AGUAS RESIDUALES INDUSTRIALES. DIGESTIÓN ANAEROBIA

La digestión anaerobia es un proceso microbiológico en el que la materia orgánica es transformada en biogás (principalmente compuesto por metano y dióxido de carbono). Esta tiene lugar en ambientes carentes de oxígeno con potenciales de reducción menores de –350 mV (Anderson y col., 2003). Este proceso ocurre en los estómagos de los rumiantes, en pantanos, sedimentos acuáticos, en vertederos municipales e incluso en el alcantarillado municipal. Algunas de las ventajas que presenta esta tecnología es su simplicidad, bajo requerimiento de espacio, baja producción de lodos en exceso y la posibilidad de emplear el metano producido como fuente de energía (van Lier y col., 2008).

1.3.1. ETAPAS Y MICROBIOLOGÍA DE LA DIGESTIÓN ANAEROBIA

La transformación de materia orgánica compleja hasta metano está constituida por diferentes etapas biológicamente complejas (Figura 1.1), que se suceden en serie (Campos y col., 2005).

1. Hidrólisis. Los compuestos orgánicos complejos como lípidos, proteínas e hidratos de carbono son transformados, por acción de enzimas hidrolíticas (celulasas, proteasas y lipasas), en moléculas solubles y fácilmente degradables, como azúcares, ácidos grasos de cadena larga, aminoácidos, alcoholes, etc. Se trata de un proceso enzimático extracelular, y las bacterias responsables de su generación son las bacterias hidrolíticas-acidogénicas (*Clostridium*, *Staphyloccocus* y *Bacteroides*).
2. Acidogénesis. Transformación de los compuestos de la etapa anterior mediante fermentación de aminoácidos y monosacáridos y β-oxidación de ácidos grasos superiores a hidrógeno, bicarbonato y ácidos acético, propiónico y butírico

(ácidos grasos volátiles, AGV), así como a otros productos orgánicos como alcoholes y ácido láctico. Las bacterias acidogénicas más comunes son *Butyrivibrio*, *Propionibacterium*, *Clostridium*, *Bacteroides*, *Ruminococos*, *Bifidobacterium*, *Lactobacillus*, *Streptococos* y *Enterobacterias*.

3. Acetogénesis. Oxidación de productos orgánicos reducidos a acetato, hidrógeno y bicarbonato por la acción de las bacterias acetogénicas productoras obligadas de hidrógeno. Como ejemplos de bacterias acetogénicas cabe mencionar *Syntrophobacter wolinii*, que descompone el ácido propionico, o *Syntrophomonas wolfei*, que descompone el butírico. Estos microorganismos requieren presiones parciales de H_2 bajas para convertir los AGV. Debido a ello, existe una estrecha relación entre acetogénicas (productoras de H_2) y metanogénas (consumidoras de H_2), ya que estas últimas ayudan a reducir la presión parcial de H_2 requerida por las acetogénicas. La presión parcial de hidrógeno juega un papel clave en la digestión anaerobia. Debe mantenerse baja (10^{-4} – 10^{-6} atm) para permitir a las bacterias acetógenicas la degradación de algunos compuestos (ácidos butítico y propiónico, etanol, etc.) que de otra forma no sería posible por tratarse de reacciones termodinámicamente desfavorables en condiciones estándar. De no existir la coordinación entre especies, la concentración de hidrógeno aumenta y la generación de acetato se inhibe. En el grupo de bacterias acetogénicas se incluyen las homoacetogénicas, capaces de producir ácido acético a partir de hidrógeno y dióxido de carbono, que pertenecen a los géneros *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Clostridium* o *Eubacterium*.

4. Metanogénesis. Constituye la etapa final del proceso donde el acetato, hidrógeno, dióxido de carbono, formiato o metanol, procedentes de la etapa anterior, son transformados en metano, dióxido de carbono y nuevo material celular. Se

distinguen dos tipos principales de microorganismos, los que degradan el ácido acético (arqueas acetoclásticas) y los que consumen hidrógeno (arqueas hidrogenotróficas).

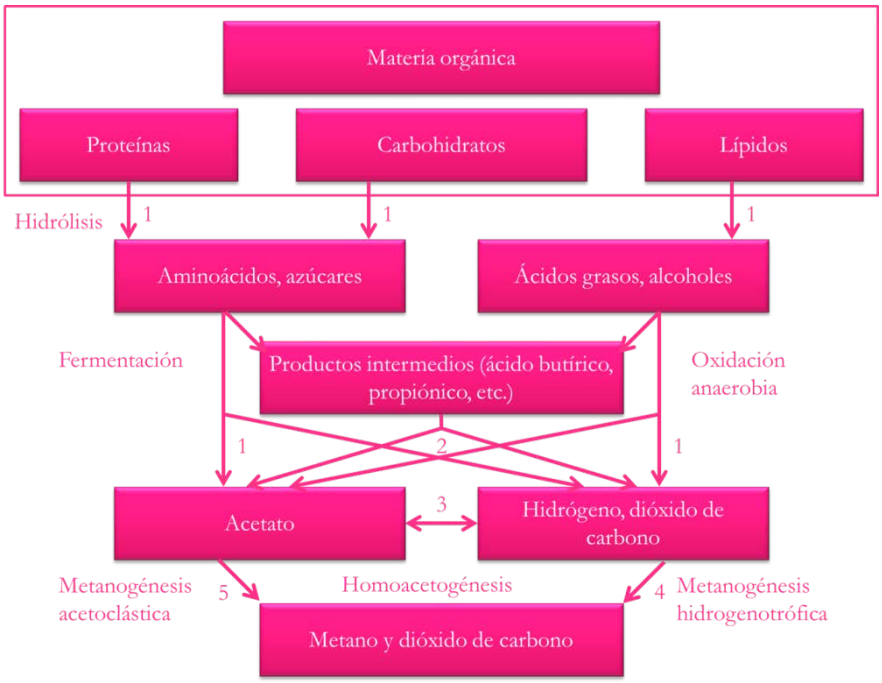

Figura 1.1. Fases de la digestión anaerobia y poblaciones bacterianas: 1) Bacterias hidrolíticas y fermentativas; 2) Bacterias acetogénicas; 3) Bacterias homoacetogénicas; 4) Arqueas metanogénicas hydrogenotróficas; 5) Arqueas metanogénicas acetoclásticas (Gujer and Zehnder, 1983).

1.3.2. METANOGÉNESIS

Los microorganismos que intervienen es las etapas anteriores forman una cadena trófica compleja y equilibrada operando de manera consecutiva y sinérgica en la transformación de substratos orgánicos complejos. En esta última etapa tiene lugar la reducción de dióxido de carbono, usando H_2 como donante de electrones, y la descarboxilación de acetato para obtener metano (Weiss y col., 2009). Las arqueas metanogénicas son anaerobios obligados que solo pueden emplear como fuente de carbono y energía acetato, H_2, CO_2, formiato y otros compuestos con un átomo de carbono como metanol y metilaminas

(Thauer, 1998). Crecen lentamente, con tiempos de generación desde 3 d a 35 °C hasta 50 d a 10 °C (Britton, 2005).

Dependiendo del sustrato que empleen para producir metano las arqueas se pueden dividir en dos grupos (Tabla 1.4):

- Metanógenas acetoclásticas. Emplean acetato para formar metano. Son responsables del 70 % del metano producido durante la digestión anaerobia. Crecen lentamente, con tiempos de generación de entre 3,5 y 9 d (Anderson y col., 2003). A pesar de ser la vía más importante, solo microorganismos pertenecientes a los géneros *Methanosaeta* y *Methanosarcina* son capaces de producir metano a partir de acetato (Campos y col., 2005). *Methanosaeta* sp. Se caracteriza por una morfología filamentosa, formando conglomerados. Solo pueden utilizar acetato para transformarlo en metano. Cinéticamente, se caracteriza por una baja velocidad máxima de crecimiento (μ_{max}) y una alta afinidad por el sustrato (van Lier y col., 2008). *Methanosaeta* sp. es el grupo dominante en reactores anaerobios cuando la concentración de acetato y AGVs es baja, indicando que el reactor opera de forma estable (Mc Hugh y col., 2003; Karakashev y col., 2005) y el tratamiento de aguas residuales está siendo efectivo. *Methanosarcina* sp. tiene forma cocoide (Subramanyam y col., 2013). El rango de sustratos que puede emplear para producir metano es más amplio que en el caso anterior, pudiendo emplear acetato, H_2/CO_2, metilaminas, metanol y formiato. Se caracterizan por una alta μ_{max} y una baja afinidad por el sustrato (van Lier y col., 2008). Estas predominan sobre las anteriores cuando la concentración de acetato en el medio es alta (Smith and Ingram-Smith, 2007) debido a que su crecimiento es más rápido (Anderson y col., 2003).

- Metanógenas hidrogenotróficas. Estas arqueas pueden reducir CO_2, formiato, metanol y metilaminas usando el H_2 producido en las etapas anteriores (Anderson y col., 2003). Producen solo el 30 % del metano en el proceso de digestión anaerobia. Crecen más rápido que las acetoclásticas, con tiempos de generación de 4 a 12 horas (van Lier y col., 2008). Los géneros más representativos son *Methanobrevibacter* y *Methanobacterium* (Macarie y Guyot, 1995).

Tabla 1.4. Reacción y parámetros cinéticos de la ruta acetoclástica de producción de metano (van Lier y col., 2008).

Reacción	ΔG^o	μ_{max}	t_d	K_s
	kJ/mol	L/d	d	mgCOD/L
$CH_3COO^- + H_2O \rightarrow$	–31	0,12[a]	5,8[a]	30[a]
$CH_4 + HCO_3^-$		0,71[b]	1,0[b]	300[b]

(a) *Methanosarcina* sp.
(b) *Methanosaeta* sp.

1.4. FACTORES AMBIENTALES E INHIBICIÓN DE LA METANOGÉNESIS

Dada la naturaleza biológica del proceso de depuración los factores que afectan a la digestión anaerobia lo hacen porque afectan a sus poblaciones microbianas.

1.4.1. O_2 y potencial redox

Muchos de los grupos bacterianos presentes en la digestión anaerobia son anaerobios facultativos por lo que la presencia de O_2 no les afecta demasiado. Sin embargo, las arqueas metanogénicas son anaerobias estrictas cuya actividad se inhibe incluso con pequeñas cantidades de O_2. No obstante, cuando se encuentran formando agregados con otros grupos bacterianos se sitúan en su interior y pueden tolerar P_{O2} relativamente altas. Del mismo modo, el potencial redox debe ser suficientemente bajo para asegurar el desarrollo de las poblaciones metanogénicas estrictas. Las arqueas metanogénicas requieren

potenciales de oxidación-reducción inferiores a –350 mV (Anderson y col., 2003).

1.4.2. pH

En cada fase del proceso los microorganismos presentan máxima actividad en un rango de pH diferenciado: hidrolíticos entre 7,2 y 7,4; acetogénicos entre 7 y 7,2 y metanogénicos entre 6,5 y 7,5. En el mantenimiento del pH es de vital importancia el sistema formado por las diferentes formas del carbono inorgánico, en equilibrio (dióxido de carbono, bicarbonato, carbónico). En algunas aguas residuales con bajo poder tampón puede llegar a ser necesario controlar externamente el pH, a fin de evitar su bajada debida a los ácidos generados en la etapa acetogénica. No es así para los residuos orgánicos complejos, como los ganaderos o los municipales, para los cuales su alta alcalinidad permite una autorregulación permanente del pH (Campos y col., 2005).

1.4.3. Temperatura

Es uno de los principales factores que influyen sobre la digestión anaerobia, ya que influye en la conversión, la estabilidad, calidad del efluente, eficacia en la producción de metano (Sánchez y col., 2001), actividad de los microorganismos, estructura microbiana y mecanismo de degradación de materia orgánica (Dhaked y col., 2010). El proceso de digestión anaerobia puede realizarse en tres rangos diferentes de temperatura (Figura 1.2):

- Psicrofílico: $5 < T < 20$ °C. Aguas residuales urbanas.
- Mesofílico: $20 < T < 40$ °C. La temperatura más empleada en aplicaciones industriales se encuentra en el rango 35-40 °C.
- Termofílico: $45 < T < 65$ °C.

Al elevar la temperatura se aumenta la tasa de hidrólisis, la velocidad de crecimiento de las bacterias y con ello la velocidad de producción

de biogás (Campos y col., 2005) y también la inhibición (Labaut y col., 2013). Por lo tanto, las principales ventajas del tratamiento en condiciones termofílicas son: mayor eficiencia en la degradación de materia orgánica, mayor producción de biogás y, además, se consigue la eliminación de microorganismos patógenos (Leven y col., 2007). Sin embargo, bajo condiciones termofílicas los reactores son menos estables, lo que puede ser debido a una menor diversidad microbiana (Leven y col., 2007), acumulación de propionato (Speece y col., 2006) y a un aumento de la toxicidad debido a los intermedios generados (Labaut y col., 2013), además de requerir más energía para alcanzar condiciones termofílicas (Leven y col., 2007).

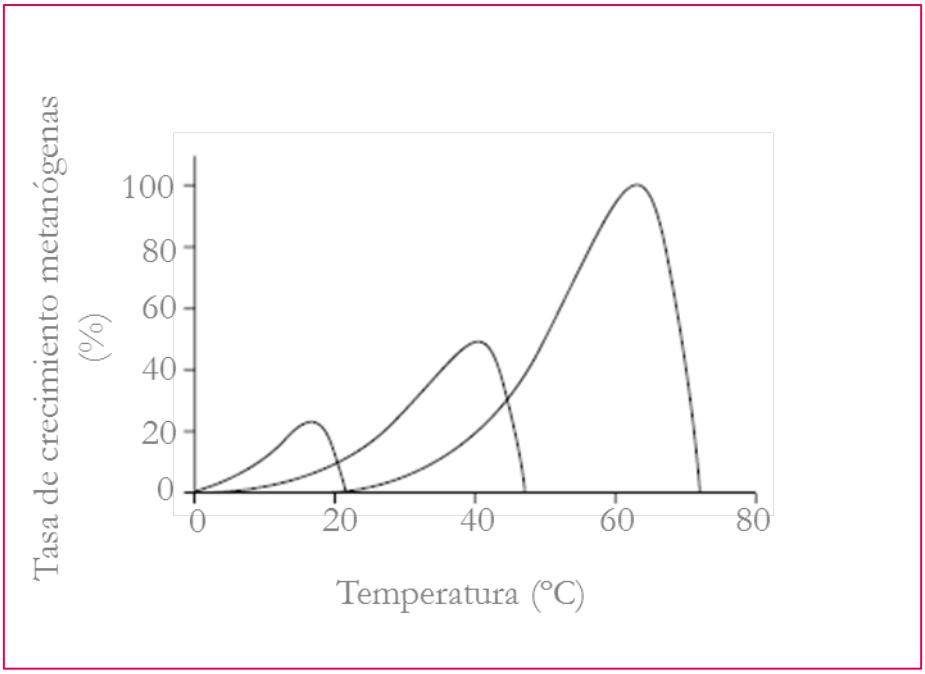

Figura 1.2. Tasa de crecimiento de arqueas metanógenas en función de la temperatura.

El mecanismo de producción de metano también es sensible a la temperatura. En condiciones psicrofílicas la metanogénesis acetoclástica es la principal ruta de producción de metano, mientras

que a altas temperaturas predomina la ruta hidrogenotrófica (Dhaked y col., 2010). Del mismo modo, Conrad (2002) establece que:

- A bajas temperaturas (en torno a 10 ºC), la formación de acetato esta favorecida frente a la de propionato, por lo que el metano se produce principalmente a partir de acetato (85 %) y solo una pequeña parte se formaría a partir de H_2/CO_2 (15 %).
- A temperaturas intermedias (alrededor de 30 ºC) un 33 % del metano producido procede del H_2/CO_2.
- A altas temperaturas (aproximadamente 50 ºC) el metano es producido exclusivamente a partir de H_2/CO_2, mientras que el acetato no es consumido y por lo tanto se acumula.

Un aumento de la temperatura de operación conlleva una menor diversidad de arqueas metanógenas. Existe mayor diversidad de arqueas en condiciones psicrofílicas que en mesofílicas. *Methanosaeta* predomina a bajas temperaturas mientras que *Methanosarcina* prevalece en condiciones mesofílicas (Dhaked y col., 2010). En condiciones mesofílicas se pueden encontrar metanógenas acetoclásticas, hidrogenotróficas y metilotróficas, sin embargo al aumentar la temperatura a 55 ºC todas las metanógenas son hidrogenotróficas (Leven y col., 2007).

1.4.4. Nutrientes

En el medio debe existir una relación de nutrientes adecuada para el desarrollo de los microorganismos. Una de las ventajas de la digestión anaerobia es la baja necesidad de nutrientes, derivada de su pequeña velocidad de crecimiento. Estos nutrientes, que pueden clasificarse en macro o micronutrientes, poseen una serie de funciones (Tabla 1.5) dentro de la digestión anaerobia.

Los principales nutrientes necesarios para el crecimiento de los microorganismos son carbono, nitrógeno y fósforo. La relación C/N

debe ser 15-30:1 y para el fósforo la relación óptima (C/P) debe estar comprendida entre 75-113:1 (Speece, 1987).

1.4.5. Tiempo de residencia hidráulico y velocidad de carga orgánica

El TRH (tiempo de permanencia del agua residual en el reactor) debe ser el adecuado para permitir el crecimiento de la biomasa y que esta no sea lavada del reactor, y para garantizar un contacto suficiente entre la biomasa y el agua residual para alcanzar eficacias altas de eliminación de DQO y producción de metano. La velocidad de carga orgánica (cantidad de materia orgánica introducida en reactor por unidad de tiempo y volumen de reactor) debe ser la óptima para alcanzar la máxima producción de metano sin causar inhibición de la metanogénesis.

Cambios bruscos en estos parámetros pueden causar sobrecarga en el sistema, provocando la acidificación del reactor por una acumulación de AGVs, y la consiguiente inhibición de la metanogénesis (Graef y Andrews, 1974; Leitao y col., 2006; van Lier y col., 2008).

1.4.6. Substancias tóxicas en la digestión anaerobia

Los procesos de digestión anaerobia pueden ser inhibidos en mayor o menor grado por la presencia de sustancias tóxicas en el sistema. Estas sustancias pueden ser productos intermedios generados por las reacciones metabólicas de las bacterias del digestor (que pueden acumularse y exceder la capacidad tampón del reactor) o sustancias que acompañan a la alimentación (Stronach y col., 1986).

El efecto sinérgico o antagónico que la presencia de una sustancia puede tener sobre la actividad tóxica de otra, juega un papel importante en el momento de definir concentraciones críticas. Del mismo modo es importante la capacidad de adaptación y aclimatación de los sistemas anaerobios, es decir, que los microorganismos puedan llegar a tolerar la presencia de compuestos tóxicos después de un período de exposición a los mismos (Campos y col., 2005).

Tabla 1.5. Funciones de los nutrientes en la digestión anaerobia (Kayhanian y Rich, 1995)

	Nutriente	Función
Macronutrientes	Carbono, C	Energía y material celular
	Nitrógeno, N	Síntesis de proteínas
	Fósforo, P	Síntesis de ácidos nucleicos
	Potasio, K	Aumenta la permeabilidad de la pared celular
	Azufre, S	Componente de varias enzimas como la monóxido de carbono deshidrogenasa (CODH) y la formiato deshidrogenasa (FDH)
Micronutrientes	Cobalto, Co	Presente en enzimas específicas (CODH) y corrinoides
	Cobre, Cu	Componente de la súper oxido dismutasa (SODH) e hidrogenasa
	Hierro, Fe	Forma parte y ayuda a la activación de numerosas enzimas. Precipita sulfuros. Promueve la excreción de polímeros extracelulares
	Molibdeno, Mo	Presente en FDH
	Níquel, Ni	Componente de COHD. Síntesis del factor F_{430}. Ayuda a la conversión de H_2/CO_2
	Selenio, Se	Componente de enzimas y ácidos nucleicos bacterianos anaerobios (FDH). Ayuda a metabolizar ácidos grasos
	Tungsteno, W	FDH. Es posible que intervenga en el metabolismo de H_2/CO_2
	Zinc, Zn	FDH, SODH e hidrogenasa

1.4.6.1. Ácidos grasos volátiles

La inestabilidad de un reactor anaerobio se manifiesta generalmente por un rápido incremento en la concentración de los AGV, lo que indica un fallo en las poblaciones metanogénicas debido a sobrecarga (cambios en la OLR o en el THR), presencia de tóxicos, cambios de temperatura o de sustrato. La acumulación de AGV refleja un desequilibrio entre los microorganismos productores de ácidos y los que los consumen (Ahring y col., 1994). El resultado de la acumulación de ácidos provoca el agotamiento de la capacidad amortiguadora y la consecuente caída del pH (Siegert y Banks, 2005). Al disminuir el pH los ácidos se encuentran en sus formas no disociadas, que son las realmente toxicas, ya que atraviesan fácilmente la membrana citoplasmática. Dentro de la célula el pH es neutro por lo que estas especies se disociarían, aumentando la concentración de protones en el interior, lo que conlleva una disminución del pH intracelular. Para expulsar esos protones en exceso y mantener el gradiente de los mismos debe existir un flujo de protones hacia afuera de la célula, lo que consume energía y afecta al crecimiento y mantenimiento del microorganismo (Fukuzaki y col., 1990).

1.4.6.2. Sulfuros

Las aguas residuales que contienen sulfato se generan en procesos en los que se usa o bien ácido sulfúrico, sulfato o compuestos de azufre reducidos como el sulfuro, tiosulfato o ditionita (Hulshoff Pol y col., 1998). En condiciones anaerobias las bacterias sulfato-reductoras utilizan el sulfato u otros compuestos oxidados del azufre como aceptor final de electrones, para la degradación de compuestos orgánicos obteniéndose como producto sulfuro (Muyzer y Stams, 2008). Las bacterias sulfato reductoras compiten con las arqueas metanogénicas por los mismos sustratos (hidrógeno, formiato y acetato), mostrando las primeras ventajas cinéticas sobre las metanógenas. Además, hay que tener en cuenta otros factores que pueden influir en esta competición como: la concentración de sulfato, el número de bacterias, pH, temperatura, concentración de sulfuro, concentración y tipo de sustrato (Oude Elferin y col., 1994) y

concentración de micronutrientes (Hidalgo y García Encina, 2001). El sulfuro producido por las bacterias sulfato reductoras en altas concentraciones es toxico para muchos microorganismos. El efecto inhibitorio del sulfuro se debe a la forma no ionizada ya que solamente las moléculas neutras pueden atravesar la membrana celular. El sulfuro puede interferir en el metabolismo asimilatorio del azufre afectando al pH intracelular. Los metanógenos son más sensibles a la inhibición por sulfuro que los acidógenos y los acetógenos (Hulshoff Pol y col., 1998). La concentración de sulfuro que provoca inhibición sobre los diferentes grupos involucrados en la producción de metano se encuentra entre 80–100 mg/L de H_2S disuelto y 50–430 mg/L de H_2S no ionizado (Parkin y col., 1990).

1.4.6.3. Amoniaco

El amoniaco se produce por degradación biológica de materia nitrogenada. Las dos principales formas de nitrógeno amoniacal que provocan inhibición sobre la digestión anaerobia son el ion amonio (NH_4^+) y el amoniaco libre (NH_3), siendo este último el más inhibitorio. La inhibición depende de las características del sustrato, pH, temperatura, inoculo, tipo de reactor y de la concentración de amonio y amoniaco (Yenigun y Demirel, 2013). Se han propuesto algunos mecanismos para la inhibición por amoniaco, como el cambio del pH intracelular, aumento de la energía requerida para el mantenimiento de la biomasa o la inhibición de reacciones enzimáticas (Whittmann y col., 1995). Las concentraciones a partir de las cuales este compuesto resulta inhibitorio varían mucho de unos autores a otros. Chen y col. (2008) recoge en su revisión que la concentración de nitrógeno amoniacal total que provoca la reducción de un 50 % de la producción de metano se encuentra en el rango 1,7–14 g/L.

1.4.6.4. Metales pesados

Muchos metales pesados forman parte de enzimas y coenzimas que intervienen en la digestión anaerobia. Sin embargo, concentraciones mayores a las requeridas para llevar a cabo la actividad biológica

pueden causar inhibición o incluso toxicidad (Chen y col., 2013; Mudhoo y Kumar, 2013). La inhibición depende de diferentes factores como el tipo de metal, la concentración del metal en disolución, su forma iónica, cantidad y distribución de la biomasa en el digestor, tipo de reactor, sustrato utilizado, pH y potencial redox (Mudhoo y Kumar, 2013). El efecto toxico esta atribuido a la disrupción de la actividad enzimática debido a la unión de los metales con los tioles u otros grupos presentes en proteínas o por reemplazamiento de un metal perteneciente a una enzima por otro (Chen y col., 2008). Otros mecanismos de inhibición/toxicidad son: i) sustitución de cofactores enzimáticos metálicos, ii) combinación con el grupo –SH, iii) inactivación del grupo mercapto de la coenzima M en metanógenas y iv) unión a grupos ácidos de los aminoácidos en la cadena de polipéptidos (Chen y col., 2014).

Los metales pesados se encuentran en aguas residuales industriales y en aguas residuales urbanas, siendo los más comunes cobre (Cu), zinc (Zn), plomo (Pb), mercurio (Hg), cromo (Cr), cadmio (Cd), hierro (Fe), níquel (Ni), cobalto (Co) y molibdeno (Mo) (Altas, 2009). En la Tabla 1.6 se muestran diferentes IC_{50} para diferentes metales pesados.

1.4.6.5. Iones metálicos ligeros

Las sales son necesarias para el crecimiento de los microorganismos, sin embargo elevadas concentraciones de las mismas pueden provocar inhibición severa o toxicidad (Soto y col., 1993) debido a cambios en la presión osmótica (de Baere, 1994, Yerkes y col., 1997). La toxicidad está determinada por el catión (Mc Carty y McKinney, 1961). Además, la presencia de un catión puede disminuir la toxicidad de otro catión, dando lugar a un efecto antagónico (Kugelman y McCarty, 1965). Los principales cationes que pueden interferir en la digestión anaerobia son:

- Aluminio. Puede afectar al crecimiento bacteriano, ya que es capaz de competir con hierro y manganeso y además puede adherirse a la pared celular (Cabirol y col., 2003). Jackson-

Moss y Duncan (1991) establecieron que concentraciones mayores de 2.500 mg/L de aluminio causan inhibición sobre la metanogénesis.

- Calcio. Puede causar precipitación de carbonato y fosfato dando lugar a incrustaciones en reactores, tuberías y biomasa, provocando una disminución de la actividad metanogénica específica. Además, puede ocasionar pérdida de capacidad tampón y de nutrientes (Chen y col., 2008). Kugelman y McCarty (1964) observaron inhibición sobre la metanización de ácido acético a partir de 2.500 mg/L de Ca^{2+}.

- Magnesio. Una elevada concentración de Mg^{2+} causa desintegración de los gránulos y estimula la producción de células individuales, siendo su concentración optima 720 mg/L (Amani y col., 2010).

- Potasio. Una de las razones por las que el K^{+} es inhibitorio está relacionada con la presión osmótica que regula el flujo de agua hacia dentro y hacia fuera de la célula. Pudiendo provocar en condiciones extremas turgencia o plasmólisis celular (Carucci y col., 2005). Mouneimne y col. (2003) establecieron una IC_{50} de 0,74 mol/L para los microorganismos consumidores de acetato.

- Sodio. Un alto contenido en sodio lleva a una disminución del tamaño del gránulo debido a que este sustituye al calcio en la matriz del gránulo (Jeison y col., 2008). El calcio une los polímeros extracelulares haciendo que las células se unan entre sí para formar los gránulos (Liv y col., 2003). Feijoo y col. (1995) establecieron una IC_{50} entre 2 y 16 g/L de Na^{+} para lodo granular anaerobio.

Tabla 1.6. Valores de IC_{50} para diferentes metales pesados sobre la producción de metano (Fuente Altas, 2009).

Fuente de carbono	IC_{50} (mg/L)					
	Cd	Cr	Cu	Ni	Zn	Pb
Glucosa	36	27	-	35	7,5	-
AGV	330	250	130	1600	270	8000
Almidón	>550	630	158	118	97	-
Benzoato	150	210	175	100	110	-
AGV	7,7	-	12,5	-	16	62,7

1.4.6.6. Compuestos orgánicos

Existe un amplio rango de compuestos orgánicos que pueden inhibir la digestión anaerobia: alquilbencenos, bencenos halogenados, nitrobencenos, fenoles y alquilfenoles, fenoles halogenados, nitrofenoles, alcanos, compuestos alifáticos halogenados, alcoholes, alcoholes halogenados, aldehídos, éteres, cetonas, acrilatos, ácidos carboxílicos, aminas, nitrilos, amidas, piridinas y sus derivados, ácidos grasos de cadena larga, surfactantes y detergentes. La concentración que causa inhibición/toxicidad depende del tipo de compuesto toxico, su concentración, cantidad de biomasa, tiempo de exposición, edad del lodo, alimentación al reactor, aclimatación y temperatura. La acumulación de estos contaminantes en la membrana celular provoca la alteración del gradiente iónico a través de la membrana causando finalmente lisis celular (Chen y col., 2008).

1.4.6.7. Aguas residuales

Las aguas residuales contienen diferentes compuestos que pueden afectar a la digestión anaerobia. La compleja composición de estas aguas puede dar lugar a que el efecto inhibitorio/toxico de un compuesto aumente o disminuya en presencia de otros compuestos, dando lugar a efectos sinérgicos o antagónicos.

Los principales compuestos que contienen las aguas residuales que pueden afectar a la digestión anaerobia se encuentran recogidos en la Tabla 1.7.

Tabla 1.7. Principales compuestos inhibitorios que contienen las aguas residuales reales. (Chen y col., 2008).

Origen	Tipo	Toxico
Agropecuario	Desechos animales	Amoniaco, sulfato, antibióticos y desinfectantes
	Cultivos	Productos fitosanitarios
Doméstico	Aguas residuales	Amoniaco
	Lodo	Metales pesados
Industrial	Conservera	Na^+, Cl^- y SO_4^{2-}
	Lechera	Intermedios de degradación: glicerol, AGCL y amoniaco
	Cárnica	Intermedios de degradación: amoniaco, AGCL, biocidas y desinfectantes
	Papel y pulpa	Sulfuro, taninos, ácidos resínicos, AGCL, y compuestos halogenados
	Textil	Tintas, surfactantes, compuestos orgánicos halogenados y metales pesados
	Petroquímica	Aldehídos, ácidos, alcoholes y ésteres

1.5. REACTORES ANAEROBIOS

El tratamiento anaerobio de aguas residuales industriales fue investigado por primera vez por Arthur M. Buswell en la década de 1920 (van Lier y col., 2015). En los sistemas estudiados el tiempo de retención hidráulico (TRH) era igual al tiempo de retención de sólidos (TRS). La eficiencia del proceso depende del crecimiento microbiano. Dado que el crecimiento de los microorganismos anaerobios es lento

los reactores requerían un TRS elevado para mantener una concentración suficiente de microorganismos efectivos dentro del reactor, lo que conlleva a elevados TRH y por consiguiente grandes volúmenes de reactor (Bal y Dhagat, 2001). Por lo que para aumentar la capacidad de tratamiento se hizo necesario desacoplar el TRS y el TRH, es decir, aumentar la concentración de biomasa disminuyendo el volumen del reactor con la reducción de costes que llevaría asociado. El primer reactor anaerobio que separaba el TRS del TRH fue el reactor anaerobio de contacto (Bal y Dhagat, 2001), dando lugar a la aparición de los reactores anaerobios de alta eficacia.

1.5.1. REACTORES ANAEROBIOS DE ALTA EFICACIA

Los reactores de alta eficacia se basan en tres aspectos fundamentales:

- Acumulación, dentro del reactor, de la biomasa por sedimentación, unión a partículas sólidas (fijas o móviles) o recirculación. Tales sistemas permiten retener microorganismos que crecen muy lentamente, asegurando que el TRS sea mucho mayor que el TRH.
- Mejora del contacto entre la biomasa y el agua residual, solucionando los problemas de difusión de substratos o productos entre el líquido y la biopelícula.
- Mejora de la actividad de la biomasa mediante adaptación y crecimiento (Lettinga, 1995).

Existen diferentes tipos de reactores anaerobios de alta eficacia dependiendo del mecanismo de crecimiento/retención de la biomasa (Figura 1.3):

- Reactores en los que los microorganismos crecen formando biopelículas sobre un soporte inerte, entre los cuales se incluyen: filtro anaerobio (AF, Anaerobic Filter) y reactores de

lecho fluidizado y reactores de lecho expandido (FB/EB, Fluidized Bed/Expanded Bed).

- Reactores en los que la biomasa es de crecimiento libre o suspendido. El tipo de crecimiento depende de que los microorganismos formen gránulos o flóculos en el reactor, para no ser lavados con el efluente. Dentro de estos se encuentran: reactor de contacto (ACP, Anaerobic Contact Process), reactor de manto de lodo de flujo ascendente (UASB, Upflow Anaerobic Sludge Blanket), reactor de manto de lodo expandido (EGSB, Expanded Granular Sludge Bed), reactor de recirculación interna (IC, Internal Circulation), reactor con deflectores (ABR, Anaerobic Baffled Reactor) y reactores de membrana (AnMBR, Anaerobic Membrane Bioreactors).

Los reactores tipo UASB y EGSB son los más extendidos en todo el mundo para el tratamiento de aguas residuales industriales (Figura 1.4), ya que constituyen aproximadamente el 90 % de la cuota de mercado de todos los sistemas instalados (van Lier, 2008).

En 2015, en el sector industrial, los reactores UASB y EGSB siguen siendo los que cuentan con un mayor número de unidades instaladas (Figura 1.5).

Los reactores de contacto se utilizan sobre todo en industrias azucareras, alcoholeras, fabricación de helado y para desechos procedentes de la agricultura. Los sistemas AnMBR son adecuados para corrientes residuales que contienen una concentración de sólidos en suspensión totales alta, y una concentración moderada de grasas y aceites. Los reactores tipo UASB se emplean en las industrias de producción de bebidas, cerveceras, papel y pulpa y hortalizas. Para los lixiviados de vertederos, industria química y producción de bebidas comúnmente se emplean los filtros anaerobios. La tecnología de lecho

fluidizado se utiliza sobre todo en las plantas de fabricación de cerveza, papel y productos químicos (Totzke, 2015).

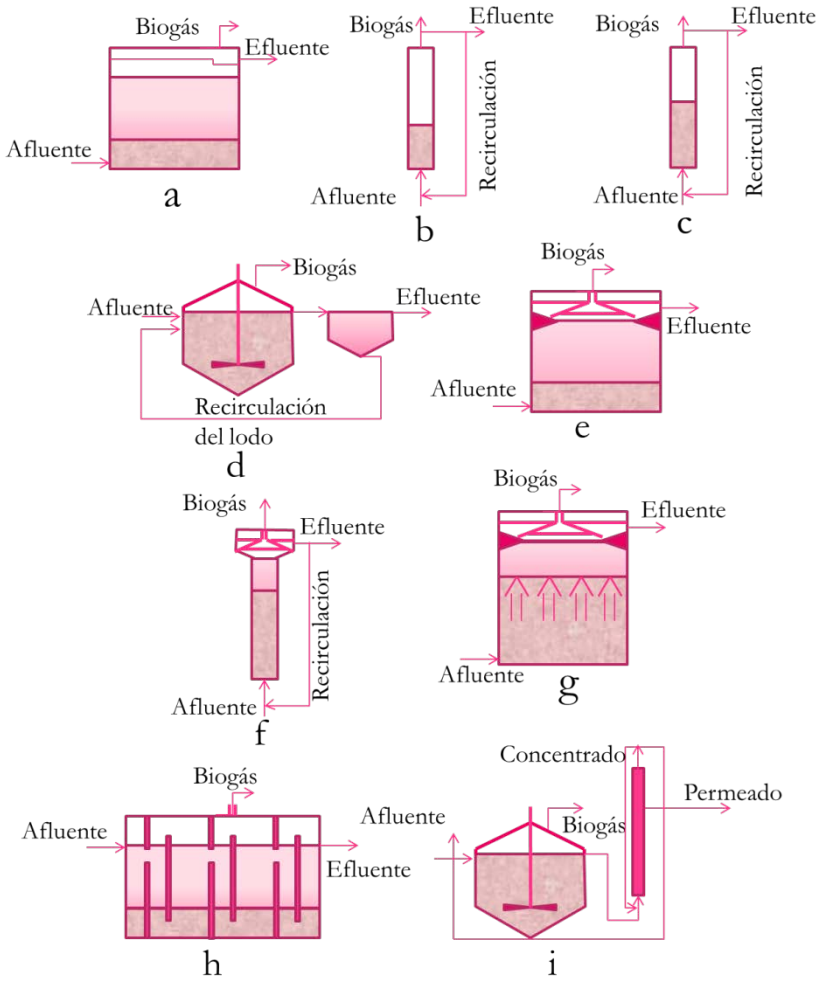

Figura 1.3. Principales tecnologías anaerobias de alta eficiencia. (a) Filtro anaerobio. (b) Reactor anaerobio de lecho fluidizado. (c) Reactor anaerobio de lecho expandido. (d) Reactor anaerobio de contacto. (e) Reactor anaerobio de manto de lodos de flujo ascendente. (f) Reactor anaerobio de manto de lodos expandido. (g) Reactor anaerobio de recirculación interna. (h) Reactor anaerobio con deflectores. (i) Reactor anaerobio de membrana.

1.5.1.1. Reactor UASB

El reactor UASB fue desarrollado a finales de la década de los 70 por el Prof. Gazte Lettinga (Lettinga y col., 1976; 1980). Su éxito se debe a la alta retención de sólidos (biomasa) dentro del reactor y a una eficiente separación de las fases sólido, líquido y gas gracias al empleo de un separador interno gas-líquido-sólido.

La retención de una elevada concentración de biomasa se fundamenta en la agregación de microorganismos anaerobios en densos y compactos gránulos, adoptando la estructura de lodo granular, la cual posee una elevada velocidad de sedimentación y una alta actividad metanogénica. Existen diferentes teorías sobre la granulación, pero la mayoría coinciden en que el primer paso es un proceso físico-químico de adhesión bacteriana. También existen evidencias de que las partículas inertes juegan un papel importante en el proceso de granulación, ya que sirven como soporte de adhesión, y que *Methanosaeta concilii* es un microorganismo clave en la granulación. Otro factor determinante para la granulación es la presión selectiva (debida a la velocidad ascensional y a la ascenso del biogás) que favorece la permanencia en el reactor de los microorganismos que se agrupan (gránulos más pesados) y elimina las formas más dispersas y floculentas, que son lavadas del reactor (Hulshoff y col., 2004).

En la Figura 1.6 se muestra un esquema de este tipo de reactores. El agua residual se introduce por el fondo del reactor y fluye a través del manto de lodo que ocupa aproximadamente la mitad del volumen del reactor. El manto de lodo está formado por gránulos anaerobios. El tratamiento tiene lugar por contacto del agua residual con los gránulos que transforman la materia orgánica en biogás y nuevos microorganismos. El biogás formado asciende hacia la parte superior del reactor arrastrando consigo agua y partículas sólidas como lodo y restos de sólidos. En la parte superior del reactor se encuentra el separador de fases (liquido-solido-gas), que permite separar internamente la biomasa, el efluente tratado y el biogás producido.

Este actúa como colector de biogás, a la vez que evita que la biomasa salga del reactor.

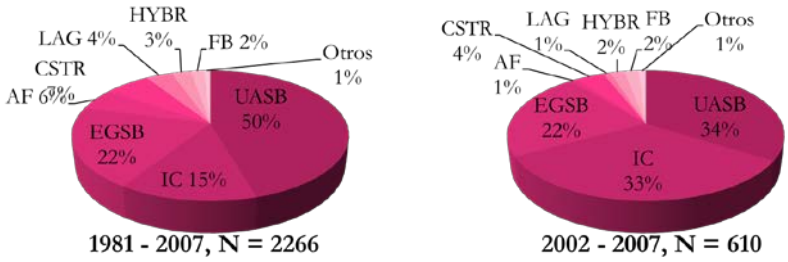

Figura 1.4. Tecnologías anaerobias instaladas para el tratamiento de aguas residuales industriales en el periodo 1981-2007 (derecha) y en periodo 2002-2007 (izquierda). UASB: reactor de manto de lodo de flujo ascendente; EGSB: reactor de manto de lodo expandido; IC®: reactor de recirculación interna; AF: filtro anaerobio; CSTR: reactor de mezcla completa; LAG: laguna anaerobia; HYBR: sistema híbrido con manto de lodo en la parte inferior y un filtro en la parte superior; FB: reactor de lecho fluidizado (van Lier, 2008).

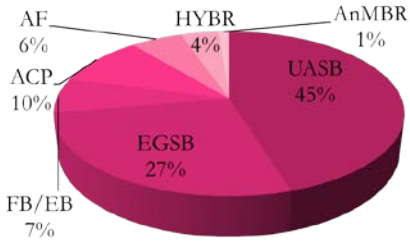

Figura 1.5. Número de reactores anaerobios de alta eficacia instalados en septiembre de 2015 (Totzke, 2015).

El funcionamiento del reactor se basa en los siguientes conceptos:

- El lodo anaerobio tiene una excelente sedimentabilidad, lo que hace no sea lavado del reactor aun aplicando elevadas velocidades ascensionales.
- El adecuado contacto existente entre el lodo y el agua residual a tratar se debe fundamentalmente a que la alimentación se

introduce por el fondo del reactor, de forma más o menos uniforme, y a la agitación causada por la producción de biogás.

- El colector de gas se comporta como un separador trifásico gas-líquido-sólido. Este separador constituye una parte esencial del reactor UASB, empleado para:
  - Colectar, separar y descargar el biogás.
  - Reduce la turbulencia en el líquido en la parte superior del reactor favoreciendo la sedimentabilidad del lodo.
  - Eliminación de partículas por sedimentación, flotación o bien por que queden atrapadas en el lodo.
  - Limita la expansión del manto de lodo.
  - Reduce o previene la flotación del lodo y por lo tanto que este sea lavado del reactor.
  - Separa la materia en suspensión del agua residual contribuyendo de esta manera al tratamiento de la misma. (van Lier y col., 2008).

Un tipo de aguas residuales industriales tradicionalmente tratadas por esta clase de tecnologías son las producidas por la industria alimentaria, debido principalmente a la elevada carga orgánica y los altos contenidos en sólidos en suspensión volátiles (SSV). A este respecto, la tecnología UASB ha sido utilizada recientemente en el tratamiento de aguas residuales de matadero (Ruiz y col., 1997; Manjunath y col., 2000; Caixeta y col., 2002; Torkian y col., 2003), industria láctea (Nadais y col., 2005; Coelho y col., 2007; Tawfik y col., 2008; García y col., 2008), cervecera (Cronin y col., 1998; Ahn y col., 2001; Rao y col., 2007), producción de café (Bello-Mendoza y Castillo-Rivera, 1998), helados (Hu y col., 2008), aceite (Angelidaki y col., 2002; Azbar y col., 2009; El-Gohary y col., 2009), industrias vinícolas (Chamy y col., 2007) y pesqueras (Puñal y Lema, 1999; Palenzuela-Rollon y col., 2002), entre otras.

Las aguas residuales de la industria papelera se caracterizan por una elevada DQO, debido a la celulosa, y por contener altas concentraciones de resinas ácidas tóxicas y compuestos orgánicos clorados, procedentes del blanqueo de la pulpa de papel. Son por ello difíciles de tratar en reactores biológicos convencionales. En cambio, han sido profusamente tratadas mediante tecnologías basadas en los reactores UASB (Kim y col., 2003; Mahadevaswamy y col., 2004; Chinnaraj y Venkoba Rao, 2006).

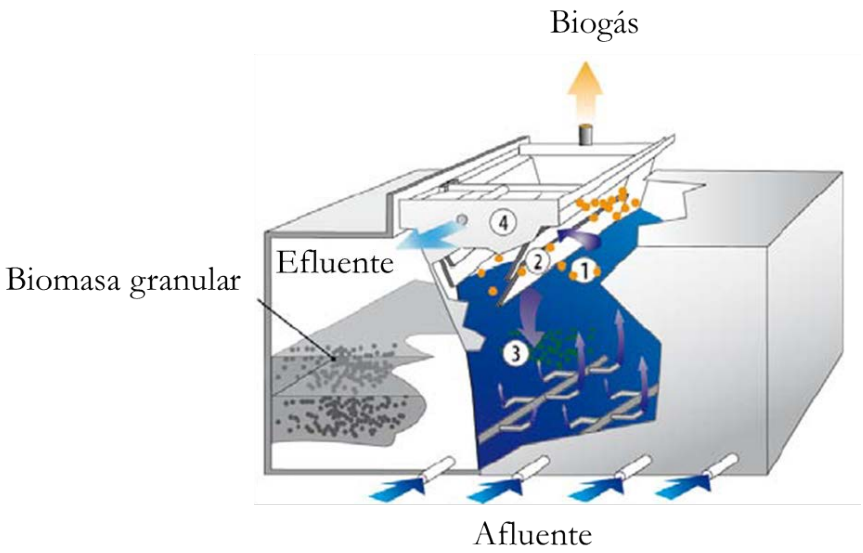

Figura 1.6. Esquema del reactor BIOTHANE®UASB (Veolia Water Solutions and Technology) para el tratamiento de aguas residuales con alta carga orgánica (10-15 kgDQO/L·d). (1) Entrada al separador de fases. (2) Placa deflectora de gas. (3) Retorno de la biomasa granular. (4) Decantador.

Otros tipos de industrias en los que la tecnología UASB ha sido un agente activo en la depuración de sus aguas residuales son la industria petroquímica (Stergar y col., 2003), farmacéutica (Zheng y Hu, 2002; Oktem y col., 2008; Sreekanth y col., 2009), textil (Gomes y col., 2007; Somasiri y col., 2008), de curtidos (Rajesh Banu y Kaliappan, 2007; Suthanthararajan y col., 2007) y del procesado de madera (Vidal y Díez, 2005), entre otras.

1.5.1.2. Reactor EGSB

El reactor EGSB es una modificación del reactor UASB en el que la introducción de una recirculación externa del efluente, combinada con una elevada relación altura/diámetro, dan lugar a una elevada velocidad ascensional. Esta alta velocidad superficial del líquido (> 4 m/h) causa la expansión del manto de lodo, haciendo un uso más eficiente del volumen del reactor, ya que se eliminan las zonas muertas y se mejora el contacto entre el agua residual y la biomasa. Comparado con el UASB, se pueden tratar cargas orgánicas más elevadas, por lo que la producción de biogás es mayor, aumentando así la mezcla interna en el reactor (Seghezzo y col., 1998). Al contrario del UASB, el EGSB retiene todo el lodo empleado, mientras que solo partículas de pequeño tamaño inactivas serian lavadas del sistema (van Lier y col., 2008).

En la Figura 1.7 se muestra un esquema del reactor BIOBED®EGSB de Veolia Water Solutions and Technology para el tratamiento de aguas residuales con cargas orgánicas muy altas (15-35 kgDQO/L·d).

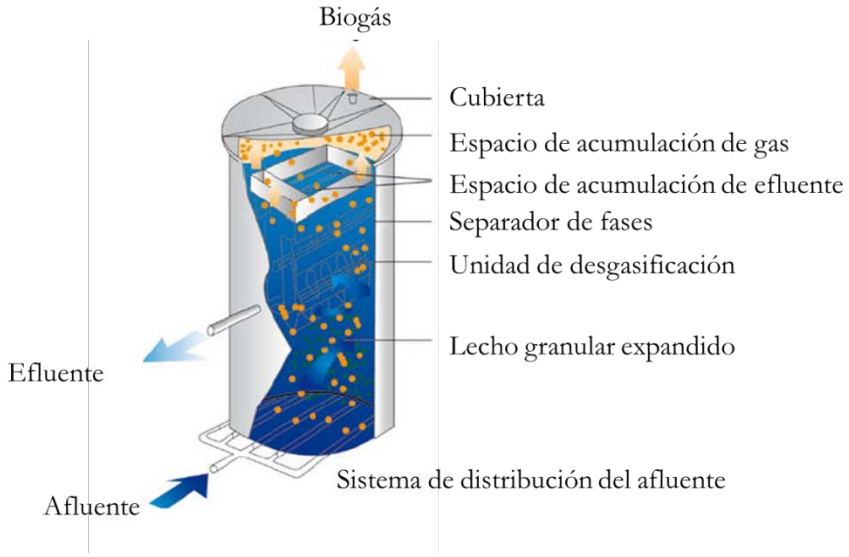

Figura 1.7. Esquema del reactor BIOBED®EGSB (Veolia Water Solutions and Technology).

El reactor EGSB se puede emplear para:

- El tratamiento de aguas residuales industriales de diversa procedencia, llegando a alcanzar velocidades de carga orgánica mayores de 40 kgDQO/m^3·d (Seghezzo y col., 1998).
- Aguas residuales que contienen compuestos tóxicos pero biodegradables como los ácidos láurico y cáprico, ácidos grasos de cadena larga, formaldehido y compuestos complejos presentes en aguas residuales industriales procedentes de la fabricación de la pasta de papel (Lettinga, 2010). La recirculación diluye la concentración del influente, lo cual permite el tratamiento de tóxicos en el reactor.
- Aguas residuales a baja temperatura (< 10 ºC) y de baja carga (DQO ≈ 200 mg/L) (Lettinga y col., 2001, Lettinga 2010).
- Aguas residuales que ocasionan problemas debido a la formación de espumas durante su tratamiento empleando un reactor UASB (van Lier y col., 2008).

Algunos ejemplos de aguas residuales industriales tratadas mediante reactores EGSB se recogen en la Tabla 1.8.

Tabla 1.8. Condiciones de operación y eficacia de tratamiento de diferentes aguas residuales industriales tratadas en reactores EGSB.

Agua residual industrial	OLR (kgDQO/m³·d)	T (ºC)	TRH (d)	Eliminación DQO (%)	Referencia
Proceso de fermentación de levadura	5	33	1	90	Wu y col., 2015
Lavandería	0,5	30	1,6	90–91	Delforno y col., 2014
Medicina china tradicional	4–10 13 20	30	0,5 0,375 0,25	90 94 78	Li y col., 2014
Lixiviado de plantas de incineración de RSU	3–18,1 16,6–19,9 17,7–18,4	33	2,5 3 4	93,2 93,2 87	Dang y col., 2012
Fracción líquida de estiércol de cerdo	0,44–2,76	30	3,8	70	López-Fernández y col., 2011
Procesado de aceite de palma	1,45–17,5	35	2	91	Zhang y col., 2008
Vinazas	20	30	1,8	89	Chamy y col., 2007

Teñido y estampado	2,14	-	0,625	35,4	Wang y col., 2007
Suero de la leche	0,5–1,3 5 –13,3	20–12	48–18	71,1–85,7 52,3–91,9	McHugh y col., 2006
Matadero	15	35	0,21	67	Nuñez y Martínez, 1999
Cervecera	11–16,5 7,5–12,6	30–12 20	1,2 2,1–1,2	35–73 81–86	Kato y col., 1998
Malteado	4,4–8,8 8,8–14,6	16 20	0,1 0,1–0,06	56 66–72	Rebac y col., 1996

1.6. OBJETIVOS

El agua es un recurso cada vez más escaso y su distribución no es equitativa, por lo que existen zonas donde los recursos hídricos son insuficientes. En este contexto, la reutilización de aguas urbanas e industriales se perfila también como un objetivo necesario para mejorar el aprovechamiento del agua como recurso natural.

La contaminación de las aguas causada por determinados contaminantes o grupos de contaminantes representa un riesgo significativo para el medio natural y el ser humano. Por ello, es preciso identificar las causas de la contaminación y desarrollar un tratamiento eficaz, en términos económicos y ambientales, para su eliminación teniendo en cuenta las condiciones en las que estas sustancias se encuentran en las aguas residuales.

En las aguas de lavado del proceso de reciclado de aceites usados aparecen en concentración significativa hidrocarburos aromáticos constituidos principalmente por numerosos derivados bencénicos y del tolueno. Estos compuestos pueden ser degradados mediante fotocatálisis (Saien y Nejati, 2007), Fenton y foto-fenton (Coelho y col., 2006), degradación fotoquímica (Wols y Caris, 2012) y ozonización (Kulik y col., 2006). Además, los hidrocarburos aromáticos pueden ser degradados biológicamente de forma aerobia (Reardon y col., 2000; Nicholson y Fathepure, 2003) y anaerobia bajo condiciones desnitrificantes (Evans y col., 1991), sulfato reductoras (Sun y Cupples, 2011) y metanogénicas (Herrmann y col., 2010).

Las aguas residuales producidas en el lavado de tanques de fabricación y envases de pesticidas contienen elevadas concentraciones de estos compuestos. Se ha demostrado que los procesos de oxidación avanzada (POAs) son un método eficaz para la eliminación de pesticidas. Entre ellos se incluyen los procesos de degradación fotoquímica (O_3/UV y H_2O_2/UV) (Andreozzi y col., 2003; Chelme-Ayala y col., 2010), fotocatálisis (TiO_2/UV), Fenton y foto-Fenton

(Legrini y col., 1993: Fallmann y col., 1999; Kitsiou y col., 2009), ozonización (Reynolds y col., 1989), oxidación con ultrasonidos (Stavarache y col., 2002) y procesos de oxidación electroquímica (Brillas y col., 2003). Además, diferentes tecnologías biológicas han sido empleadas en la biodegradación de pesticidas en condiciones aerobias (Sanchis y col., 2013) anóxicas (Stasinakis y col., 2009) y anaerobias (López y col., 2013).

Además de las aguas residuales anteriormente citadas, las aguas residuales generadas en el lavado químico de instalaciones industriales, son el objetivo principal de la presente Tesis Doctoral. Estas últimas se caracterizan por contener compuestos que actúan como inhibidores de corrosión, entre los que se han seleccionado como compuestos modelo: benzotriazol, quinoleína, morfolina y piperazina. Estos compuestos son considerados tóxicos y mutagénicos. Además, la morfolina y la piperazina pueden formar N-nitrosaminas (potentes agentes mutagénicos y cancerígenos), por lo que estos xenobióticos son de especial interés desde el punto de vista ambiental. Estos inhibidores de corrosión pueden ser eliminados por degradación fotoquímica (Andreozzi y col., 1998) oxidación con ozono molecular y radicales hidroxilo (Nathalie y Babak, 2009), oxidación húmeda (Mishra, 1994), procesos tipo Fenton (Prousek, 2004), procesos fotocatalíticos (Doherty y col., 1995) y mediante tratamiento térmico (Freeman y Rochelle, 2011). Se ha demostrado que estos compuestos pueden ser degradados por microorganismos aerobios (Jianlong y col., 2002: Sun y col., 2009; Liu y col., 2011; Ali, 2011) y bajo diferentes condiciones anaerobias (Li y col., 2010; Liu y col., 2011; Liu y col., 2013).

Como se puede observar las técnicas de tratamiento de aguas residuales más utilizadas engloban los procesos químicos y biológicos. El principal inconveniente de los POAs es su alto coste de operación, lo que dificulta su implantación a escala industrial. Además, en algunos casos el tratamiento no consigue reducir la toxicidad del agua residual. Otras opciones más económicas son el empleo de la radiación solar

para la generación del radical hidroxilo o la combinación de POAs con sistemas biológicos (Zapata el al., 2010). Entre los sistemas biológicos el tratamiento anaerobio se perfila como una alternativa económicamente viable, no solo desde el punto de vista de reducción de costes de los POAs, sino también por que consumen menos energía que los sistemas aerobios, originan menor cantidad de fangos y producen un biogás que puede utilizarse como combustible. Entre las diferentes tecnologías anaerobias que pueden aplicarse para el tratamiento de estos compuestos se encuentran los sistemas anaerobios de alta eficacia, entre los que destaca el reactor anaerobio de lecho expandido, ya que puede emplearse para el tratamiento de aguas toxicas y complejas.

El objetivo de la presente Tesis Doctoral se centra en evaluar la aplicación de reactores anaerobios de alta eficacia para el tratamiento de contaminantes recalcitrantes y tóxicos (plaguicidas e inhibidores de corrosión) que pueden estar presentes en diferentes efluentes industriales, así como su aplicación al tratamiento de aguas residuales industriales reales de diferente procedencia, como las aguas del proceso de reciclado de aceites industriales usados y las aguas lavado de tanques de fabricación de productos fitosanitarios. La consecución del objetivo general descrito se desarrollará a través de los siguientes objetivos específicos:

Biodegradabilidad de inhibidores de corrosión bajo condiciones anaerobias

- Estudio de la biodegradabilidad anaerobia y del posible efecto inhibitorio sobre la actividad metanogénica del lodo granular de diferentes inhibidores de corrosión (benzotriazol, quinoleína, morfolina y piperazina) empleando distintas concentraciones iniciales de los mismos.

- Determinación de la viabilidad del tratamiento de aguas sintéticas contaminadas con los compuestos anteriormente

citados mediante un reactor anaerobio de alta eficacia en términos de eliminación de materia orgánica, reducción de la concentración de inhibidores de corrosión y producción de metano. Así mismo, se estudiará la evolución de las bacterias anaerobias y de las arqueas metanogénicas presentes en el reactor durante el tratamiento mediante el empleo de técnicas de secuenciación.

Eliminación biológica anaerobia de pesticidas comerciales

- Evaluación de la puesta en marcha, aclimatación y eficacia de tratamiento de un agua residual que contiene una mezcla de pesticidas comerciales cuyos compuestos activos son ácido 2-metil-4-cloro-fenoxiacético, imidacloprid y dimetoato mediante el empleo de un reactor EGSB que opera a velocidades de carga de diferentes pesticidas entre 20 y 100 mg/L·d. Se estudiarán los cambios producidos en la microbiota a lo largo del tratamiento mediante el análisis de la morfología del gránulo y de la identificación de las diferentes arqueas metanogénicas presentes en el reactor.

- Estudio de la capacidad de degradación de los pesticidas seleccionados empleando lodo granular anaerobio y de la posible toxicidad que pueden causar sobre las arqueas acetoclásticas e hidrogenotróficas. Se identificarán los intermedios de degradación para el establecimiento de las rutas de degradación. Así mismo, se evaluara el efecto producido por diferentes mezclas de pesticidas (binarias y ternarias) sobre la biodegradación individual de cada uno de ellos.

Viabilidad del tratamiento anaerobio de un agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios

- Evaluación de la biodegradabilidad anaerobia de un agua residual procedente del lavado de tanques de fabricación de

productos fitosanitarios empleando diferentes concentraciones de la misma y análisis de la respuesta de las arqueas acetoclásticas e hidrogenotróficas en presencia del agua residual industrial.

- Tratamiento de aguas residuales que contienen productos fitosanitarios mediante un reactor anaerobio de alta eficacia. Se evaluara la eficacia de tratamiento, la composición y biodegradabilidad aerobia de los efluentes obtenidos del tratamiento anaerobio en condiciones mesofílicas (35 ºC) y termofílicas (55 ºC). Así mismo, se determinarán los cambios producidos en las poblaciones microbianas como consecuencia del aumento de la temperatura de operación.

Tratamiento biológico de un agua residual industrial procedente del reciclado de aceites industriales usados mediante un reactor anaerobio de alta eficacia

- Determinación de las condiciones de operación optimas de un reactor EGSB que trata un agua residual industrial procedente del reciclado de aceites industriales usados. Para ello, se estudiará la influencia de la temperatura (17-32 ºC) y de la velocidad de carga orgánica (0,5-10,5 gDQO/L·d) sobre la eficiencia del proceso y sobre las poblaciones microbianas que intervienen en el mismo.

- Evaluación de la biodegradabilidad anaerobia del agua residual objeto de estudio aplicando distintas concentraciones iniciales, lo que permitirá establecer el intervalo de concentración recomendable a emplear. También se determinará la influencia que este tipo de aguas residuales tiene sobre la producción de metano del lodo granular por vía acetoclástica e hidrogenotrófica.

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2

MATERIALES Y MÉTODOS

2. MATERIALES Y MÉTODOS

2.1. FUENTE DE BIOMASA

Se empleó lodo granular anaerobio procedente de un reactor UASB instalado en una planta de tratamiento de aguas residuales de una industria azucarera situada en Valladolid.

2.2. BIODEGRADABILIDAD ANAEROBIA E INHIBICIÓN / TOXICIDAD ANAEROBIA

La medida de la actividad metanogénica específica (SMA, Specific Methanogenic Activity), biodegradabilidad e inhibición/toxicidad se realizó empleando el analizador automático de potencial metano (AMPTS, Automatic Methane Potential Test System) desarrollado por Bioprocess Control AB (Lund, Sweden).

El sistema se divide en 4 unidades: A, B, C y D. En la unidad A, se realiza la incubación de las muestras en 15 frascos de 0,5 L con lodo granular anaerobio (1,5 gSV/L) a una temperatura dada (35 ºC), la cual se mantiene constante en todos los ensayos en discontinuo. El contenido de cada frasco se mezcla mediante un agitador rotatorio lento. El biogás que se produce se utiliza para indicar la actividad de biometanización dentro de cada frasco. En la unidad B (fijación de CO_2), el biogás producido en cada frasco se hacer pasar a través de un frasco individual (100 mL) que contiene una solución de NaOH (3 M). Se eliminan varias fracciones de gas, tales como CO_2 y H_2S, debido a las reacciones químicas que sólo permiten al CH_4 pasar a través de la solución de álcali. En cada frasco se añade también un indicador de pH (timolftaleína 0,4 % w/v) para controlar la disolución con el fin de garantizar una concentración de OH^- suficientemente alta para la fijación de CO_2 y H_2S. En la unidad C (monitoreo del metano), el gas CH_4 liberado de la unidad B se analiza utilizando un dispositivo de medición de flujo de gas mediante celdas multi-flujo (15 celdas). Este dispositivo de medición funciona de acuerdo con el principio de desplazamiento líquido y puede monitorear un flujo de gas ultra bajo,

donde un pulso digital se genera cada vez que un volumen dado de gas fluye a través del dispositivo. Un sistema de adquisición de datos (unidad D) se utiliza junto con las celdas de flujo con el fin de grabar, mostrar y calcular los datos, así como analizar los resultados.

El ensayo de SMA se realizó empleando como fuente de carbono 4 gDQO/L de una disolución de ácidos grasos volátiles (ácidos acético, propiónico y butírico, 1:1:1, w:w:w, 440 gDQO/L). Además, se añadieron 20 mL/L de los siguiente macronutrientes (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328), $MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10), extracto de levadura (4) y 1 mL/L de la siguiente disolución de micronutrientes (μg/L): $FeCl_2 \cdot H_2O$ (2.000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2.000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO \cdot 5H_2O$ (162), EDTA (1.000), resazurina (0,2) y ácido sulfúrico 36 % (1 μL/L). Como fuente de alcalinidad y para mantener el pH en el intervalo 7-7,5 se empleó 4 $gNaHCO_3$/L. Para desplazar el oxígeno del espacio de cabeza y asegurar condiciones anaerobias se burbujeo N_2 durante 5 min. Estos ensayos se realizaron por triplicado y se mantuvieron hasta el agotamiento de la fuente de carbono. Los valores de SMA se estimaron ajustando los datos de producción de metano a un pseudo-primer orden, Modelo de Roediger (Edeline, 1980).

La biodegradabilidad anaerobia se llevó a cabo de forma similar a los ensayos de SMA pero sustituyendo los ácidos grasos volátiles por distintas fracciones del agua residual objeto de estudio (entre 4–32 gDQO/L del agua residual procedente de la recuperación de aceites industriales usados y entre 1,2–9,1 gDQO/L del agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios) o añadiéndole diferentes concentraciones de inhibidores de corrosión y pesticidas (10–500 mg/L) como única fuente de carbono. La duración del ensayo se extendió alrededor de 30 d para las aguas industriales reales y entre 21 y 45 d para los inhibidores de corrosión y los pesticidas comerciales. La biodegradabilidad se

determinó a partir de la evolución de la DQO, COT, concentración del compuesto objeto de estudio y de la producción de metano.

La inhibición metanogénica acetoclástica e hidrogenotrófica se determinó de forma independiente mediante ensayos en discontinuo en las mismas condiciones que los ensayos de SMA. Se utilizó como fuente de carbono acetato de sodio (4 g/L) o formiato de sodio (2 g/L) para estimular la biomasa acetoclástica e hidrogenotrófica, respectivamente, además de adicionar diferentes fracciones del agua residual correspondiente (entre 0,125–2 gDQO/L del agua residual procedente de la recuperación de aceites industriales usados y entre 1,8 (0,05)–12,8 (2,53) gDQO/L del agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios). En el caso de inhibidores de corrosión y pesticidas comerciales se procedió en primer lugar a la estimulación de la actividad acetoclástica e hidrogenotrófica del lodo, mediante la adición de acetato de sodio o formiato de sodio como única fuente de carbono, respectivamente. Posteriormente, empleando el mismo lodo granular se realiza una nueva adición de acetato/formiato de sodio junto con diferentes inhibidores de corrosión o pesticidas (10–500 mg/L) para estudiar la inhibición. Finalmente, se vuelve a adicionar acetato de sodio o formiato de sodio como única fuente de carbono para estudiar si la inhibición era reversible o irreversible (toxicidad).

Entre los diferentes pasos se lavó el lodo empleando una disolución de $NaHCO_3$ (4 g/L) para asegurar que en el medio solo se encuentran los compuestos que se añaden en cada paso y no compuestos o intermedios del paso anterior. Estos ensayos se mantuvieron hasta el agotamiento del acetato de sodio o formiato de sodio o hasta que finalizó la producción de metano. La inhibición se evaluó comparando la producción de metano en ausencia y presencia del agua residual o de los inhibidores de corrosión y los pesticidas.

2.3. BIODEGRADABILIDAD AEROBIA

El estudio de biodegradabilidad aerobia del agua residual procedente del lavado de los tanques de fabricación de productos fitosanitarios y de los efluentes resultantes del tratamiento anaerobio a diferentes temperaturas (35 y 55 ºC) se realizó mediante respirometría, utilizando fangos activos procedentes de un reactor secuencial discontinuo (SBR, Sequencial Batch Reactor) que era alimentado con acetato sódico y glucosa (1:1 en DQO) como fuentes de carbono. El procedimiento está basado en el empleo de condiciones de operación próximas a las utilizadas en los procesos de fangos activos, con la finalidad de determinar si los efluentes objeto de estudio podrían eliminarse mediante un sistema biológico de fangos activos. Estos ensayos de biodegradabilidad rápida se llevaron a cabo utilizando un respirómetro discontinuo de medida en fase líquida (LSS, Liquid-Static-Static) (Chica et al., 2007). El aporte de aire en el sistema depende de dos valores, máximo y mínimo, de concentración de oxígeno disuelto en el medio de reacción, que deben establecerse lo suficientemente elevados para impedir limitación de oxígeno en el mismo. Se inyecta aire hasta llegar al valor máximo, y una vez alcanzado, se detiene la aereación y se registra la disminución de la concentración de oxígeno disuelto debida al metabolismo microbiano, hasta llegar al valor mínimo, a partir del cual comienza de nuevo la aereación en un proceso cíclico. La disminución de oxígeno disuelto en el tiempo por unidad de biomasa determina la velocidad específica de consumo de oxígeno (VECO).

El respirómetro utilizado consta de dos reactores independientes para operar de manera simultánea y garantizar la reproducibilidad de los resultados a través de ensayos por duplicado, realizados a temperatura fija y constante empleando un baño termostatizado. Los recipientes de reacción de vidrio de 1 L se encuentran cerrados herméticamente para poder descartar la transferencia de oxígeno del aire a la fase líquida, conteniendo en su interior las sondas de oxígeno y las conducciones de aire, ambas controladas mediante una interfase que registra la

concentración de oxígeno a tiempo real, y un agitador magnético para homogeneizar el medio de reacción.

En el ensayo de biodegradabilidad rápida se puso en contacto el agua residual procedente del lavado de los tanques de fabricación de productos fitosanitarios y los efluentes resultantes del tratamiento anaerobio a diferentes temperaturas (35 y 55 ºC) con fangos activos (3.500 mgSSV/L), medio mineral (APHA, 1992) y tampón fosfato, durante 24 h a 30 ºC de temperatura. La biodegradabilidad se determinó a partir de la evolución del COT, DQO y el perfil de VECO obtenido a lo largo del experimento.

2.4. INSTALACIÓN EXPERIMENTAL

El lodo empleado en los ensayos en discontinuo se mantuvo activo en un reactor UASB y los experimentos en continuo se realizaron en un reactor EGSB. Ambos reactores tienen un volumen de 5,2 L, una relación diámetro-altura de 1:7,2 y poseen una camisa exterior para mantener constante la temperatura durante la experimentación. Además, están provistos de un separador de gas-sólido-líquido situado a 15 cm de la parte superior de los reactores. La alimentación se introduce mediante bombas peristálticas, así como la recirculación en el caso del reactor EGSB. El dióxido de carbono se eliminó del biogás utilizando una trampa de NaOH (4 M) tipo Mariotte, cuantificando el metano generado con un gasómetro hidráulico. Las muestras se tomaron en la parte superior del reactor, se filtraron y se almacenaron a –20 ºC hasta su análisis.

2.5. EXPERIMENTOS EN CONTINUO

2.5.1. Aguas sintéticas que contienen una mezcla de inhibidores de corrosión

El reactor operó aplicando una velocidad ascensional de recirculación de 2,5 m/h, en condiciones mesofílicas (35 ºC) y con un TRH de 1 d. La velocidad de carga orgánica (OLR, Organic Loading Rate) empleada fue de 4 gDQO/L·d, la cual se mantuvo constante durante

los 157 d de operación en continuo. El reactor se inoculó con 100 gSV/L de lodo granular anaerobio, el cual fue aclimatado con el agua sintética formada por un sustrato metanogénico que contenía una mezcla de glucosa (2 g/L) y 2 gDQO/L de ácidos grasos volátiles (ácidos acético, propiónico y butírico, 1:1:1, w:w:w, 440 gDQO/L). Además, se adicionó macro y micronutrientes y $NaHCO_3$ (1 g/gDQO). Una vez que el sistema alcanzó el estado estacionario se introdujeron los inhibidores de corrosión en la alimentación del reactor EGSB con una concentración de 124 mg/L de benzotriazol, 70 mg/L de quinoleína, 98 mg/L de morfolina y 98 mg/L de piperazina.

2.5.2. Aguas sintéticas que contienen una mezcla de pesticidas comerciales

El reactor operó aplicando una velocidad ascensional de recirculación de 2,5 m/h, en condiciones mesofílicas (35 ºC) y con un TRH de 1 d. La OLR empleada fue de 1,75 gDQO/L·d, la cual se mantuvo constante durante los 408 d de operación en continuo. El reactor se inoculó con 100 gSV/L de lodo granular anaerobio, el cual fue aclimatado con el agua sintética de baja carga (descrita en los capítulos 4 y 5) sin pesticidas. Además, se adicionó macro y micronutrientes y $NaHCO_3$ (1 g/gDQO). Una vez que el sistema alcanzó el estado estacionario se introdujeron los pesticidas objeto de estudio en la alimentación del reactor EGSB. En una primera etapa la concentración de pesticidas que se añadió fue la correspondiente a un 20 % del COT del agua residual sintética de baja carga. Por lo que para mantener constante la OLR al añadir los pesticidas, se disminuía la fracción de agua sintética alimentada al reactor. En la Tabla 2.1 se detallan las condiciones de operación empleadas en cada una de las etapas.

2.5.3. Agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios

El reactor se inoculó con 100 gSV/L de lodo granular anaerobio previamente activado como se indica anteriormente. El reactor operó con una velocidad ascensional de recirculación de 2,5 m/h, con un TRH de 1 d en condiciones mesofílicas (35 ºC) y termofílicas (55 ºC). Durante la etapa mesofílica el reactor operó con una OLR de 2 gDQO/L·d, correspondiente a la adición del agua residual real, durante 76 d. A partir de ahí la temperatura se elevó a 55 ºC empleando como única fuente de carbono el sustrato metanogénico (descrito en el apartado 2.5.1) para aclimatar la biomasa a condiciones termofílicas. Una vez estabilizado el reactor se substituyó el medio metanogénico por el agua residual real aplicando una OLR de 2 gDQO/L·d durante 62 d. La alimentación, formada tanto por sustrato metanogénico como por el agua residual, fue suplementada con macro y micronutrientes y $NaHCO_3$ (1 g/gDQO).

2.5.4. Agua residual procedente de la recuperación de aceites industriales usados

El reactor se inoculó con 100 gSV/L de lodo granular anaerobio previamente activado empleando un medio metanogénico (descrito en el apartado 2.5.1) hasta que se alcanzó el estado estacionario, aplicando una OLR de 4 gDQO/L·d. Se comenzó el tratamiento del agua residual procedente de la recuperación de aceites industriales usados aplicando una OLR de 0,5 gDQO/L·d para no causar inhibición sobre la biomasa. A partir de ahí, la OLR se aumentó gradualmente hasta 10,5 gDQO/L·d, mientras que el tiempo de residencia hidráulico (TRH) se redujo de 6 a 1 d. El reactor operó con una velocidad ascensional de recirculación de 2,5 m/h a temperatura ambiente (17–21 ºC) durante 67 d, momento en el cual se hizo necesario controlar la temperatura para mantener la eficacia del reactor, aumentando está a 32 ºC hasta el final del tratamiento (día 82). Durante las etapas de activación de la biomasa y de tratamiento, la alimentación fue suplementada con macro y micronutrientes y $NaHCO_3$ (1 g/gDQO).

Tabla 2.1. Condiciones de operación del EGSB tratando un agua residual de baja carga que contiene pesticidas.

Etapa	t (d)	Pesticida	Concentración (g/L)	COT (%)
I	0	MCPA	0,058	20
		Imidacloprid	0,020	
		Dimetoato	0,025	
		Ciclohexanona	0,027	
II	90	MCPA	0,087	30
		Imidacloprid	0,029	
		Dimetoato	0,038	
		Ciclohexanona	0,041	
III	285	MCPA	0,100	40
		Imidacloprid	0,039	
		Dimetoato	0,050	
		Ciclohexanona	0,054	

2.6. ELECTROFORESIS EN GEL CON GRADIENTE DESNATURALIZANTE (DGGE)

2.6.1. Preparación de la muestra

El lodo granular (0,05 g) se resuspendió en tampón fosfato (PBS, phosphate buffered saline, 130 mM de NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7,2) y se disgregó mediante el disruptor BIO101-Savant FP120 (Q-BIOgene, Carlsbad,CA, USA) 6 veces durante 40 s, frecuencia 5,5 ciclos/s.

2.6.2. Extracción de ADN

La extracción de ADN del lodo granular se realizó de acuerdo al protocolo establecido en el kit FastDNA kit for soil (Q-BIOgene, Carlsbad, CA, USA). Se toma 1 mL de lodo granular y se centrifuga 15

min a 14.000 rpm. Se resuspende el pellet conseguido con 980 µL de PBS y se mezcla con 120 µL de solución MT buffer (tampón de lisis). La mezcla obtenida se añade a los tubos de lisado matriz E, los cuales contienen esferas de diferente tamaño para romper los gránulos y extraer el ADN de forma mecánica además de química, y se introducen en el equipo FastPrep (equipo para agitación) con los siguientes parámetros: 3 ciclos de ruptura a 5,5 de potencia y 40 s. Entre ciclos se enfrían las muestras en hielo. A continuación, se centrifuga 5 min a 14.000 rpm y se transfiere el sobrenadante a un eppendorf de 1,5 mL al que se le añade 250 µL de disolución de precipitación de proteínas (PPS, Protein Precipitation Solution) y se agita invirtiendo el eppendorf varias veces lentamente. Seguidamente, se centrifuga 5 min a 14.000 rpm y se transfiere el sobrenadante a un eppendorf de 5 mL al que se le añade 1 mL de disolución de unión a la matriz (Binding Matriz Suspension), para que el ADN se adhiera a la matriz y se agita la mezcla lentamente en un rotor vertical durante 30 min a 4 ºC. Posteriormente, se filtra mediante filtro SPIN, se centrifuga a 14.000 rpm durante 1 min y se desecha el sobrenadante. A la resina contenida en el filtro se le añade 500 µL de disolución de lavado sal/etanol (SEWS M, salt/ethanol wash solution) y se centrifuga a 14.000 rpm durante 1 min. Para secar la muestra se centrifuga nuevamente el filtro SPIN a 14.000 rpm durante 3 minutos. Una vez que el filtro está seco se le añade 100 µl de disolución de agua ultra pura para la extracción de ADN (DES, DNA Elution Solution-ultra pure water) y se resuspende la resina (homogenizar durante 2 minutos). Finalmente se vuelve a centrifugar a 14.000 rpm durante 3 minutos. El líquido filtrado contiene el ADN y debe conservarse a 4 ºC.

2.6.3. Amplificación mediante la reacción en cadena de la polimerasa de un fragmento del gen del ARNr 16S para DGGE

Para amplificar mediante la reacción en cadena de la polimerasa (PCR, Polymerase Chain Reaction) fragmentos del gen del ARNr 16S a partir de una muestra de lodo granular, para realizar posteriormente DGGE, se utilizarán las condiciones establecidas en la Tabla 2.2 y los

siguientes pares de cebadores (Tabla 2.3): 341F(GC)-907R para bacterias y 622F(GC)-1492R para arqueas, siendo las temperaturas de anillamiento 52 y 42 ºC, respectivamente. Como ADN polimerasa se empleó la Taq ADN polimerasa (Promega, Madison, WIS, USA). Para llevar a cabo la amplificación por PCR se emplearon los siguientes programas: 10 min de desnaturalización a 94 ºC seguido de 35 ciclos de 1 min a 94 ºC (desnaturalización), 1 min a 52 ºC (anillamiento), 2 min a 72 ºC (elongación) y un ciclo final de 10 min a 72 ºC, para bacterias. Para arqueas el programa es el mismo pero cambiando el número de ciclos a 30 y la temperatura de anillamiento a 42 ºC.

Tabla 2.2. Condiciones de la PCR para posterior DGGE (volumen de 100 µl).

Compuesto	Cantidad
Tampón 10 X	10 µL
$MgCl_2$ (25 mM)	12 µL
dNTPs (50 mM)	1 µL
Cebadores (50mM)	1 µL de cada uno (Forward y Reverse)
Taq ADN polimerasa	0,5 µL
ADN	1-10 µL (Depende de las características de la muestra y de su concentración)
Agua MilliQ	Hasta 100 µL

Los productos de PCR se someterán a electroforesis horizontal (90 V, 1 h) en gel de agarosa (1 % w/v), que se visualizara después de teñir con bromuro de etidio, para comprobar la amplificación del fragmento rRNA 16S.

2.6.4. DGGE

Se preparó un gel de poliacrilamida al 6 % (v/v) (37,5:1 acrilamida-bisacrilamida) con un gradiente desnaturalizante de 30 a 70 % de urea-formamida (el 100 % corresponde a 7 M urea y un 40 % de

formamida desionizada (v/v)). Una vez que el gel ha polimerizado a 4 ºC se introduce en la carcasa del equipo D-Code Universal system (Bio-Rad, Hercules, CA, USA) que contiene 7,5 L de buffer TAE 1X (tris 0,04 M, ácido acético glacial 0,02 M y EDTA 0,001 M). Los productos de PCR del apartado anterior se concentran a vacío hasta reducir su volumen completamente y se resuspenden en 10 μL de tampón de carga para cargar las muestras en el gel. La electroforesis tiene lugar a 200 V y 60 ºC durante 5 h. Finalmente el gel se tiñe con bromuro de etidio y se visualiza bajo luz UV. Las bandas se cortan, se reamplifican con los mismos cebadores (pero sin la grapa GC) y se purifican de acuerdo al siguiente protocolo.

Tabla 2.3. Cebadores utilizados para la amplificación por PCR del gen ARNr 16S de Bacterias y Arqueas.

Cebador	Secuencia (de 5´a 3´)	Dominio	Referencia
907R	CCGTCAATTCMTTTGAGTTT	Bacteria	Brosius, 1981
341F-GC*	CCTACGGGAGGCAGCAG	Bacteria	Brosius, 1981
622F-GC*	TGAAATCYYRTAATCCC	Arquea	Chan, 2001
1492R	TACGGYTACCTTGTTACGACTT	Arquea	Lane, 1991

GC*: secuencia de 40 nucleótidos rica en GC, unida al extremo 5´ del cebador. La secuencia GC es: 5´-CGC CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3´. (M = C:A, Y = C:T, R = A:G).

2.6.6. Identificación de la biomasa

Las secuencias fueron ensambladas empleando el programa Chromas 2.0 para posteriormente ser comparadas con las que se encuentran en la base de datos Genbank del NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.go) empleando el programa BLAST (Basic Local Alignment Search Tool). Esta herramienta bioinformática lleva a cabo un análisis de similitud comparando la secuencia objeto de estudio con las secuencias que existen en la base de datos, encontrando aquellas secuencias que tienen mayor parecido a la secuencia problema. La jerarquía taxonómica se determinó mediante la herramienta de clasificación de la página web "Ribosomal Database Project" (RDP) (http://rdp.cme.msu.edu/index.jsp).

2.7. SECUENCIACIÓN MASIVA (Illumina)

2.7.1. Extracción de ADN y secuenciación

La extracción de ADN y la secuenciación se realizó en el laboratorio Research and Testing Laboratory (Lubbock, Texas, USA) mediante el sistema Illumina MiSeq (2x250 chemistry), empleando los cebadores 357wF-785R y 519wF-909R para la identificación de bacterias anaerobias y arqueas, respectivamente.

2.7.2. Análisis de la biodiversidad y caracterización filogenética

Tras la extracción y secuenciación masiva del ADN, los datos obtenidos se procesaron en el laboratorio Research and Testing Laboratory, eliminándose las lecturas de poca calidad y las quimeras. La limpieza de todas aquellas lecturas de poca calidad se llevó a cabo mediante el algoritmo USEARCH, dando lugar a una secuencia consenso (Edgar, 2010). La detección de secuencias quiméricas se realizó mediante el algoritmo UCHIME (Edgar y col., 2011). La secuencia resultante es revisada y agrupada en unidades taxonómicas operacionales (OTUs, Operational Taxonomic Units) a través del algoritmo UPARSE (Edgar, 2013). A continuación, cada OTU se

identifica empleando el algoritmo de alineamiento global USEARCH (Edgar, 2010) y una base de datos de secuencias de alta calidad del NCBI mantenida por el laboratorio Research and Testing Laboratory.

2.8. MICROSCOPÍA ELECTRÓNICA DE BARRIDO (SEM)

La microscopía electrónica de barrido permite la formación de la imagen a través de la amplificación electrónica de señales generadas al irradiar la superficie de la muestra con un haz muy estrecho de electrones. Esta irradiación hace que la muestra desprenda electrones de baja energía (electrones secundarios) que son recogidos sobre una placa cargada positivamente (ánodo). De este modo se produce una señal eléctrica y con ello la imagen ampliada de la muestra. Esta técnica resulta muy versátil para la visualización y análisis de las características superficiales y microestructurales de asociaciones microbianas. Se analizaron mediante SEM muestras de gránulos procedentes del reactor EGSB y del inóculo inicial con el objetivo de comparar su morfología y estructura. Los gránulos se fijaron con glutaraldehido (2,5 % w/w) durante 2 h y posteriormente se lavaron con una disolución de cacodilato sódico (0,2 M, pH 7,1) durante 30 min. El lodo se deshidrató posteriormente mediante disoluciones de concentraciones crecientes de etanol (entre 10 y 100 %) durante 20 min en cada disolución (Alphenaar et al., 1994). En un intercambiador de alta presión Emitech K850 se intercambió, durante 10 min, el etanol presente por dióxido de carbono líquido y se calentó 20 min hasta alcanzar las condiciones del punto crítico del CO_2 (80 bar y 40 ºC). En un Sputter Caoter SC502 se recubrieron las muestras con oro a 20 mA y 1 mbar durante 45 s en atmósfera de Ar y se observaron en un microscopio electrónico de barrido (Phillips XL30, Ámsterdam, Países Bajos).

2.9. MÉTODOS ANALÍTICOS

2.9.1 Carbono orgánico total

La concentración de carbono orgánico total (COT) se cuantificó mediante un equipo COT-$V_{CPH/CPN}$, de Shimadzu, que permite determinar la concentración de carbono total (CT) y carbono inorgánico (CI), calculando por diferencia el carbono orgánico total de la muestra analizada. Para la determinación del CT la muestra a analizar se oxida con aire a 680 ºC en un tubo de cuarzo relleno de catalizador de Pd/Alúmina. El CO_2 generado se cuantifica mediante un detector de infrarrojo, generando un pico cuya área es proporcional a la cantidad de carbono presente en la muestra y se integra por un procesador de datos. Para la cuantificación del CI, se inyecta una nueva fracción de muestra a un depósito donde se acidifica con ácido clorhídrico. En estas condiciones, el carbono inorgánico asociado a la presencia de carbonatos y/o bicarbonatos se desprende de la alícuota en forma de CO_2. El CO_2 generado es arrastrado por el aire de alta pureza y procesado, de la misma forma que el CT.

2.9.2. Demanda biológica de oxígeno

La determinación de la DBO_5 se llevó a cabo siguiendo el método 5210 (APHA 1992), utilizando un equipo de Velp Scientifica, que consta de una serie de sensores de DBO_5 y una incubadora. La incubación, de 5 d, se realiza a 20 ºC y en ausencia de luz para evitar la producción de oxígeno por parte de microorganismos fotosintéticos. Se añadieron micronutrientes para permitir el crecimiento microbiano y N-aliltiourea para inhibir la nitrificación. El volumen de muestra empleado fue de 100 mL. Los ensayos se realizaron por triplicado para comprobar la reproducibilidad del método.

2.9.3. Demanda química de oxígeno

La determinación de la DQO se llevó a cabo siguiendo el protocolo 5220A (APHA 1992). Este método consiste en realizar la digestión de un volumen conocido de agua residual con dicromato potásico

($K_2Cr_2O_7$) en un medio fuertemente ácido (H_2SO_4), valorándose la cantidad de oxidante remanente, tras la digestión, con un reductor (sal de Mohr). El procedimiento consiste en añadir al tubo de análisis 1,5 mL de disolución digestora de dicromato potásico (15 g/L), 3,5 mL del reactivo de ácido sulfúrico (5,5 g de Ag_2SO_4 por cada kg de H_2SO_4) y 2,5 mL de la muestra de agua residual objeto de análisis. Los tubos se agitan y se lleva a cabo la digestión de la mezcla a 150 ºC durante 2 h, en un bloque digestor Lovibond, modelo ET108. A continuación se dejan enfriar los tubos a temperatura ambiente, y finalmente se lleva a cabo la valoración del dicromato potásico sobrante, con disolución de sal de Mohr, utilizando ferroína como indicador. El punto final de la valoración se alcanza con el viraje de color verde azulado a marrón rojizo.

2.9.4. Sólidos en suspensión totales y sólidos en suspensión volátiles

Las medidas de los sólidos en suspensión totales (SST) y sólidos en suspensión volátiles (SSV) se realizaron de acuerdo a métodos estandarizados (2540 D y E, APHA, 1992).

Para la determinación de SST y SSV se emplearon filtros de fibra de vidrio (FILTER LAB) que fueron previamente lavados con agua MilliQ e introducidos en la mufla a 550 ºC durante 15 min. Posteriormente se dejan en un desecador hasta pesada constante.

En primer lugar se registra el peso del filtro utilizando una balanza de precisión. Posteriormente se filtrar un volumen determinado de la muestra problema y se introduce el filtro en una estufa (GALLENKAMP, Hotbox Oven with Fan, size 1) a 105 °C durante 1 h. Después de ese tiempo el filtro se enfrió en un desecador, para finalmente pesarlo. La diferencia entre pesadas es el valor de SST (mg/L). Para la cuantificación de los SSV se somete al filtro a 550 ºC durante 30 min en una mufla HOBERSAL (modelo 12 PR400). Posteriormente el filtro se deja enfriar en un desecador y se pesa. La diferencia con respecto a la pesada anterior son los SSV (mg/L).

2.9.5. Determinación de los sólidos totales (ST), volátiles (SV) y peso seco del lodo

Debido a que prácticamente la totalidad de los SV presentes en el lodo granular corresponden a microorganismos o restos de ellos, para determinar la cantidad de lodo granular a utilizar en los ensayos anaerobios es habitual referirse a lodo húmedo y establecer la relación másica entre el lodo húmedo y los SV. Para la determinación de los SV se utilizó el protocolo seguido en el Standard Methods (2540 E). En este método se toma una muestra de lodo granular y, tras escurrir el agua, se registra su peso, valor conocido como peso húmedo. Posteriormente se somete a 105 ºC durante 2 h. Una vez que se ha enfriado, el lodo se vuelve a pesar. La diferencia entre ambos valores son los sólidos totales o peso seco del lodo, con unidades de gST/g de peso húmedo. Finalmente, el lodo seco se calcina a 550 ºC durante 1 h en un horno mufla y, después de enfriarse, se vuelve a pesar. La diferencia con respecto a la pesada anterior son los sólidos volátiles, con unidades de gSV/g de peso húmedo.

2.9.6. Cromatografía de gases con detector de ionización de llama

La cuantificación de morfolina y piperazina se llevó a cabo mediante cromatografía de gases con detector de ionización de llama (GC/FID). El cromatógrafo de gases empleado (GC 430 Varian), con inyector (1177), utiliza una columna capilar de fase estacionaria (GC Care Column BR-Volatile amines fs, Agilent), con 30 m de longitud y 0,32 mm de diámetro interno. La inyección de 8 µL de muestra se realizó a través de un inyector automático (Autosampler CP-8410). El método utilizado se detalla en la Tabla 2.4.

La cuantificación de ciclohexanona y ciclohexanol se realizó mediante GC/FID. El cromatógrafo de gases empleado (CG 3900 Varian), con inyector (1177), utiliza una columna capilar de fase estacionaria muy polar (CP-Wax 52 CB, Varian), construida en acero inoxidable, con 30 m de longitud y 0,25 mm de diámetro interno. La inyección de 1 µL

de muestra se realizó a través de un inyector automático (Autosampler CP-8400). El método utilizado se detalla en la Tabla 2.5.

Tabla 2.4. Método utilizado para el análisis de morfolina y piperazina mediante GC/FID.

INYECTOR	Split: 20			
	T: 250 ºC			
	Caudal gas portador (N_2): 30 mL/min			
COLUMNA	T (ºC)	Rampa (ºC)	t_m (min)	t_t (min)
	40		2	2
	250	20	5	17,5
DETECTOR FID	T: 300 ºC			
	Caudal (H_2): 30 mL/min			
	Caudal (aire): 300 mL/min			

t_m: tiempo de mantenimiento
t_t: tiempo total

Tabla 2.5. Método utilizado para el análisis de ciclohexanona y ciclohexanol mediante GC/FID.

INYECTOR	Split: 20			
	T: 150 ºC			
	Caudal gas portador (N_2): 30 mL/min			
COLUMNA	T (ºC)	Rampa (ºC)	t_m (min)	t_t (min)
	70		1	1
	240	15	4,67	17
DETECTOR FID	T: 300 ºC			
	Caudal (H_2): 30 mL/min			
	Caudal (aire): 300 mL/min			

t_m: tiempo de mantenimiento
t_t: tiempo total

2.9.7. Cromatografía de gases con detector de masas

El análisis de las muestras se realizó en el laboratorio de Cromatografía del Servicio Interdepartamental de Investigación (SIdI) de la Universidad Autónoma de Madrid, mediante cromatografía de gases con detector de masas (CG/MS) empleando un cromatógrafo de gases Varian 3800 con detector de trampa iónica 4000MS. El equipo está dotado con un inyector automático CP-8200/SPME (Solid Phase Micro Extraction, microextracción en fase sólida).

Una vez obtenidas las muestras analizadas, la técnica permitió detectar e identificar cualitativamente compuestos con una relación de masa (m/z) comprendida en el intervalo 40-600 uma (unidades de masa atómica) presentes en las muestras. Los espectros de estos compuestos se compararon para la asignación de picos con los recogidos en la base de datos del equipo (Nist 05).

El agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios se analizó empleando una columna de tipo capilar (Factor Four IV) de 30 m de longitud y 0,25 mm de diámetro interno. Para llevar a cabo la microextracción en fase sólida se utilizó un cartucho de fibra PDMS roja (Polidimetilsiloxano), siendo el tiempo de adsorción de 30 min y el de desorción de 5 min. En la Tabla 2.6 se recoge el método de análisis empleado.

El agua residual procedente de la recuperación de aceites industriales usados se analizó utilizando una columna de tipo capilar (Factor Four VF-5ms) de 30 m de longitud y 0,25 mm de diámetro interno. Para llevar a cabo la microextracción en fase sólida se empleó un cartucho de fibra (Carbowax/Divinilbenceno, yellow-green), siendo el tiempo de adsorción de 20 min y el de desorción de 5 min. En la Tabla 2.7 se recoge el método de análisis empleado.

Tabla 2.6. Método de operación del CG-MS con SPME para el análisis del agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios

INYECTOR	Split: 20			
	Temperatura: 220 ºC			
	Caudal gas portador (He): 1mL/min			
COLUMNA	T (ºC)	Rampa (ºC/min)	t_m (min)	t_t (min)
	40		5	5
	250	15	10	29
	300	20	2	33,5
DETECTOR	Tiempo (min)			
MASAS	0 – 33,5			

t_m: tiempo de mantenimiento
t_t: tiempo total

Tabla 2.7. Método de operación del CG-MS con SPME para el análisis del agua residual procedente de la recuperación de aceites industriales usados.

INYECTOR	Split: 20			
	Temperatura: 220 ºC			
	Caudal gas portador (He): 1mL/min			
COLUMNA	T (ºC)	Rampa (ºC/min)	t_m (min)	t_t (min)
	40		15	15
	250	15	1	30
DETECTOR	Tiempo (min)			
MASAS	0 - 30			

t_m: tiempo de mantenimiento
t_t: tiempo total

2.9.8. Cromatografía líquida con detector de masas

El análisis de las muestras para identificar los posibles intermedios de degradación se realizó en el laboratorio de Cromatografía del Servicio Interdepartamental de Investigación (SIdI) de la Universidad Autónoma de Madrid, mediante cromatografía líquida de alta

resolución con ionización de electrospray acoplada a espectrometría de masas tipo cuadrupolo (LC-ESI-MS) (HPLC Agilent 1100 series, Analizador de masas tipo cuadrupolo modelo Agilent 6120, Agilent Technologies, Santa Clara, CA, USA). Se empleó una columna Gemini (Phenomenex) de 150 mm de longitud, 4,6 mm de diámetro interno y 5µm de tamaño de partícula. Como fase móvil se utilizó una mezcla de acetonitrilo en ácido fórmico (0,1 % v/v) en gradiente como se indica en la Tabla 2.8.

Tabla 2.8. Gradiente seguido en la determinación por LC-ESI-MS.

Tiempo (min)	Ácido fórmico (0,1 % v/v)	Acetonitrilo
0	85	15
3	70	30
6	60	40
12	50	50
15	20	80
20	85	15
50	85	15

Una vez obtenidas las muestras analizadas, la técnica permitió detectar e identificar cualitativamente los iones de acuerdo a su relación masa/carga (m/z).

2.9.9. Cromatografía líquida de alta resolución

La concentración de diferentes compuestos se cuantificó mediante cromatografía líquida de alta resolución (HPLC) utilizando un equipo Varian que dispone de un muestreador automático Prostar 410, un módulo interfase Star 800, un módulo de bombeo Prostar 230 y dos detectores (Índice de refracción (IR) modelo 350 y UV-visible Prostar 325). La Tabla 2.9 recoge los métodos y columnas empleados para la determinación de los diferentes grupos de compuestos.

Tabla 2.9. Métodos y columnas empleados en la cuantificación de compuestos analizados por HPLC.

Compuesto	Columna	Fase móvil	Caudal (mL/min)	Tiempo (min)	Detector
Etilenglicol Propilenglicol Etanol AGVs	Varian Metacarb 67H 300–6,5 mm	H_2SO_4 0,025N	0,6	30	IR
MCPA	Teknokroma Mediterranea Sea18 250–4,6 mm, 5 µm	Acetonitrilo:Agua 80-65% (15 min) 65-25% (15 min)	0,6	30	UV (220 nm)
Imidacloprid Dimetoato	Sunfire™ Waters C18 150–3 mm, 5 µm	Acetonitrilo:Agua 15-80 % 80-20 % (rampa 4 ºC/min)	0,5	18	UV (220 nm)

MCPA Imidacloprid Dimetoato	Microsorb MV 100-5 C18 250–4,6 mm	Acetonitrilo:Agua 15–80 % 80–20 % (rampa 3 ºC/min)	0,5	27	UV (MCPA y Dimetoato 220 nm, Imidacloprid 270 nm)
Benzotriazol Quinoleína	Microsorb MV 100-5 C18 250–4,6 mm	Metanol:Agua 40-60 % 90-10 % (rampa 3 ºC/min)	1	22	UV (Benzotriazol 225 nm, Quinoleína 250 nm)

2.10. BIBLIOGRAFÍA

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3

BIODEGRADATION OF CORROSION INHIBITORS UNDER ANAEROBIC CONDITIONS

3. BIODEGRADATION OF CORROSION INHIBITORS UNDER ANAEROBIC CONDITIONS

Abstract

Chemical cleaning of the industrial facilities generate effluents containing corrosion inhibitors whose toxicity makes necessary to explore technologies for their efficient removal from wastewaters. In the present work, the biodegradability and inhibitory and/or toxic effects of four corrosion inhibitors (benzotriazole, quinoline, morpholine and piperazine) were studied. Benzotriazole remained unaltered during the individual biodegradability test, while quinoline, morpholine and piperazine were partially biodegraded. Quinoline degradation rate was severely affected by the presence of other N-heterocyclic compounds. However, piperazine biodegradation rate was improved in presence of other corrosion inhibitors. Benzotriazole removal efficiency was not altered in mixtures and morpholine elimination was slightly affected. A significant toxic effect of quinoline was observed over the acetoclastic methanogens decreasing their activity by a 65 %. Hydrogenotrophic activity was slightly affected (22 %) by the presence of these compounds. However, when corrosion inhibitors were removed hydrogenotrophic activity was retrieved. A synthetic wastewater containing corrosion inhibitors was treated in an EGSB reactor. The N-heterocyclic compounds dramatically decreased the EGSB reactor performance. After 122 d of operation the system achieved constant COD removal and methane production efficiency of 80 % and 0.38 gCH_4-COD/gCOD, respectively. Quinoline and piperazine were successfully removed during the continuous experiment. The phylogenetic characterization revealed the prevalence of genus *Methanosaeta* and the phylum *Proteobacteria* at the end of the treatment.

3.1. INTRODUCTION

Corrosion protection strategies are of great importance in order to extend the useful life of materials and equipments at industrial facilities, especially in those segments such as oil and gas, power, refining, chemical, and pharmaceutical. For this purpose, corrosion inhibitors are extensively used to mitigate the impact of this phenomenon on metallic materials, creating a protective film on the corroding surface (Videla and Herrera, 2008). Significant amounts of aqueous off-streams containing anti-corrosion agents are generated, which have to be treated before discharge. Commercial inhibitors are organic compounds containing heteroatoms such as nitrogen, oxygen, sulphur and phosphor atoms (Qiu et al., 2007). Benzotriazole, quinoline, morpholine and piperazine are N-heterocyclic compounds widely used as corrosion inhibitors.

Benzotriazole is used as a corrosion inhibitor in dishwasher detergents and de-icing/anti-icing fluids, an ultraviolet light stabilizer in plastics, and an antifogging agent in photography labs and airports (Liu et al., 2011). Its fate along the wastewater treatment process remains unclear (Gruden et al., 2001). Liu et al. (2011) found benzotriazole concentrations, used as an antifogging agent, higher than 126 mg/L in groundwater, which must be well-managed because it is carcinogenic and toxic for fish, invertebrates and bacteria at relatively low concentration (Jia et al., 2006). Benzotriazole has been characterized by a slow biodegradation under aerobic (46 %) and anaerobic microcosms (microbial consortium: iron (III) reducing (31 %), nitrate reducing (24 %) and sulfate reducing (18 %)) after 91 d using activated sludge and digested sludge (Lui et al., 2011). However, these results were improved using groundwater and aquifer sediment material, obtaining removal efficiencies of 72, 67, 52 and 48 % under aerobic, iron (III) reducing, nitrate reducing and sulfate reducing conditions, respectively, after 77 d of incubation (Lui et al., 2013).

Quinoline is present in coal tar, oil, petroleum, creosote, pharmaceuticals, pesticides, dyes and corrosion inhibitors (Sun et al., 2009; Ebenso et al., 2010). This species has been detected in different industrial wastewaters, reaching concentrations between 150 and 1,000 mg/L (Pinto et al., 2006). Due to its toxic, carcinogenic and mutagenic character at low concentrations, quinoline is considered as hazardous to human health and environment (Johansen et al., 1997b; Li et al., 2010). Thus, quinoline bearing wastewater must be treating before discharge. Aerobic biodegradation of quinoline has been widely studied by several specialist degrading microorganisms like *Pseudomonas* sp., *Burkholderia* sp., *Rhodococcus* sp., *Comamonas* sp., and *Moraxella* sp. (Sun et al., 2009; Bao-hua Tuo et al., 2011). Anaerobic biodegradation of quinolines has been observed under nitrate-reducing, sulfate-reducing, and methanogenic conditions. Johansen et al., (1997a; 1997b) reported the complete degradation of quinoline (25–54 mg/L) by a biofilm mixed culture under nitrate-reducing conditions and by the sulphate-reducing bacterium *Desulfobacterium indolicum*. Battersby and Wilson (1989) observed the complete degradation of quinoline (60 mg/L) after 4 weeks of incubation under methanogenic conditions. Wang et al. (1984) also achieved the removal of quinoline from a granular activated carbon, methanogenic filter treating a synthetic feed containing quinoline (42–290 mg/L).

Morpholine is a heterocyclic compound commonly used in corrosion inhibitors, solvents, rubber additives, herbicides, drugs, optical brighteners, antioxidants and catalysts (Lamant and Jaffrin, 1996). It is well-known that secondary amines can be transformed biologically and chemically to nitrosamines, which are potent mutagens and carcinogens (Bae et al., 2002). Hence, the removal of morpholine from polluted industrial wastewaters is of great environmental and public health interest. Industrial off-streams resulted from the chemical cleaning of pipelines in power plants contain morpholine concentrations of around 1.2 g/L (Pliego et al., 2013). The most effective morpholine degrading bacteria are *Mycobacterium* sp., *Arthrobacter* sp., bacteria belonging to genus *Pseudomonas* and mixed

activated sludge (Meister and Wechsler, 1998; Schräder et al., 2000). However, biodegradation was negligible by consortium incubated under sulfate-reducing and methanogenic conditions after 6 months (Bae et al., 2002).

Piperazine is an important alicyclic amine used as corrosion inhibitor, which has been characterized as toxic and carcinogenic to humans and other living organisms (Cai et al., 2013). Piperazine has been partially biodegraded by several bacteria in the genera *Arthrobacter*, *Mycobacterium* (Adjei et al., 2007; Kim et al., 2006) and *Paracoccus* sp. which can degrade completely 100 mg/L of piperazine in 24 h (Cai et al., 2013).

Most of N-heterocyclic compounds can be mineralized by anaerobic bacteria (Liu et al., 1994), being the critical step the partial scission of polycyclic and heterocyclic rings, cleavage of long chains, and degradation of these organics through anaerobic fermentation (Li et al., 2001). In parallel, anaerobic high-rate technology has improved significantly in the last few decades, expanding their applicability to hardly-biodegradable wastewater that can contain toxic compounds or with complex composition (van Lier et al., 2015). Expanded Granular Sludge Bed (EGSB) reactors are the most widely used reactors for the anaerobic treatment of industrial wastewaters (van Lier, 2008), which have been reported to be adequate for dealing with hardly biodegradable wastewater, and to dampen the inhibition of the microbial activity caused by the presence of toxic pollutants (Tauseef et al., 2013). Thus, anaerobic treatment of N-heterocyclic compounds using an EGSB reactor is an attractive alternative and economically option for a sustainable wastewater management.

In this work, the anaerobic biological treatment of synthetic wastewater containing corrosion inhibitors by an EGSB reactor is studied. Anaerobic biodegradability and toxic effect of these compounds on the methanogenic activity of the granular sludge were also analyzed in batch tests. In addition, the evolution of the microbial

population during the long-term experiment was studied by Illumina sequencing technology.

3.2. MATERIALS AND METHODS

Wastewater composition

Synthetic wastewater was prepared to achieve a total chemical oxygen demand (COD) of around 4 g/L, by adding a methanogenic substrate composed by a mixture of acetate:propionate:butyrate (450 mg/L of each compound) and glucose (1884 mg/L). Corrosion inhibitors were incorporated as a fraction of total organic carbon (TOC) of 10 %, which corresponds to 124, 70, 98 and 95 mg/L of benzotriazole, quinoline, morpholine and piperazine, respectively. The feed was supplemented with 20 mL/L of the following macronutrient solution (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328), $MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and yeast extract (4). This medium was supplemented with 1 mL/L of the subsequent micronutrients solution (μg/L): $FeCl_2 \cdot H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO \cdot 5H_2O$ (162), EDTA (1,000), resazurin (0.2) and sulfuric acid 36 % (1 μL/L). $NaHCO_3$ (1 g/gCOD) were supplemented as buffer and alkalinity source.

Biomass source

Anaerobic granular biomass was collected from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating beet sugar wastewater (Valladolid, Spain). The granules had an average diameter of 0.5 mm and a specific methanogenic activity (SMA) of 0.269 (0.006) gCH_4-COD/gVS·d.

Anaerobic biodegradability and methanogenesis inhibition and toxicity test

Anaerobic batch tests were performed inoculating 1.5 g volatile solids (VS)/L of non-adapted granular sludge. The experiments were carried out at 30±1 °C in duplicate, using the Automatic Methane Potential

Test System (AMPTS, Bioprocess Control, Sweden) following the procedure reported elsewhere (Garcia-Mancha et al., (2012)).

Anaerobic biodegradability tests of individual and mixtures of corrosion inhibitors were performed for 27 d. Initially, corrosion inhibitors were added individually as sole carbon source at different concentrations ranging from 10 to 500 mg/L of active ingredient. Biodegradability of binary, tertiary and quaternary mixtures of N-heterocyclic compounds was evaluated at a relative concentration of 20 % of the TOC fed: benzotriazole (244 mg/L), quinoline (139 mg/L), morpholine (191 mg/L) and piperazine (190 mg/L).

The inhibition and toxic effect over acetoclastic and hydrogenotrophic methanogenesis was studied following the next three-steps method. First, the anaerobic acetoclastic and hydrogenotrophic biomass contained in the sludge was activated adding sodium acetate (4 g/L) or sodium formiate (2 g/L), respectively. Second, sodium acetate/formiate and the corrosion inhibitors (at different initial concentrations from 10–500 mg/L) were added at the same time to evaluate the inhibition caused by the presence of the N-heterocyclic compounds. Finally, in order to study the biomass recovery and therefore the possible toxic effects (irreversible inhibition) caused by the target compounds, sodium acetate/formiate were added as sole carbon source.

Abiotic test

The contribution of adsorption was evaluated in biomass samples after extraction with Soxhelt following the US-EPA 8041 method. Tests of volatilization were performed under identical operating conditions to those in the biodegradation experiments but in the absence of biomass. The results reported are the average values from duplicate runs, the standard errors being always lower than 10 %.

Experimental set-up for continuous runs

Experiments in continuous mode were carried out in a 5.2 L EGSB reactor with an internal diameter to height ratio of 1:7.2. The reactor was equipped with a gas–liquid–solid separator installed 15 cm below the exit. Details of the apparatus are described elsewhere (Monsalvo et al., 2014). The reactor was operated at an upward flow rate of 2.5 m/h, mesophilic condition (35 °C) and a hydraulic retention time (HRT) of 1 d. The EGSB reactor was inoculated with 100 gVS of granular sludge previously activated adding the synthetic feed without spiking the target compounds until reaching the steady stage. Macro- and micronutrients, as well as $NaHCO_3$ (buffer and alkalinity source) were supplemented as indicated above. The organic loading rate (OLR) of 4 gCOD/L·d were maintained constant during the 157 d of the continuous experiment. N-heterocyclic compounds were added as a 10 % of the TOC of simulated wastewater (124 mg/L of benzotriazole, 70 mg/L of quinoline, 98 mg/L of morpholine and 98 mg/L of piperazine).

DNA extraction and sequencing

In order to study the shifts in bacteria and archaea communities along the anaerobic processes Illumina sequencing technology was performed. For this purpose, granular sludge was extracted from the EGSB reactor at the beginning and at the end of the continuous run. DNA was extracted and sequenced on an Illumina MiSeq (2x250 chemistry) system by Research and Testing Laboratory (Lubbock, Texas, USA). The primers used for bacteria were 357wF-785R and for archaea were 519wF-909R.

Biodiversity analysis and phylogenetic classification

Once sequencing was completed, the data obtained was processed and denoised and chimeras were removed from the data set at Research and Testing Laboratory. The denoising was performed using USEARCH algorithm for dereplication and clustering (4 % divergence), resulting in the consensus sequence and the elimination of poor reads (Edgar, 2010). Chimera checking was performed on the

selected operational taxonomic units (OTUs) using the UCHIME chimera detection software executed in de novo mode (Edgar et al., 2011). The remaining sequence must be checked and clustered into OTUs using the UPARSE algorithm (Edgar, 2013). Then, each OTU is identified using the USEARCH global alignment algorithm (Edgar, 2010) and a database of high quality sequences derived from NCBI maintained by Research and Testing Laboratory. The USEARCH method rejects the taxonomic information at a level if the confidence is below 51 %. After resolving the number of sequences per OTU, the percentage of each organism was individually calculated for each sample. Relative abundance was defined as the number of sequences affiliated with that specie divided by the total number of sequences per sample.

Analytical methods

Analyses of total and soluble COD, total and volatile suspended solids (TSS, VSS) were performed according to the APHA Standard Methods (APHA, 1992). TOC was measured by a Shimadzu TOC-$V_{CPH/CPN}$ analyzer.

The quantification of benzotriazole and quinoline was performed by high-performance liquid chromatography (HPLC) (Varian Prostar 325, Santa Clara, CA, USA) with a UV detector using a reverse-phase column (Agilent Technologies, Microsorb MV 100-5 C18 250×4.6 mm). The mobile phase was methanol (40 %) and H_2O (60 %) in a concentration gradient to 90 % of methanol and 10 % of H_2O in 22 min (flow rate 1 mL/min). A wavelength for benzotriazole of 225 nm and 250 nm for quinoline was used. Morpholine and piperazine were quantified by gas chromatography with a flame ionization detector (GC 430 Varian, Santa Clara, CA, USA) using a 30 m long×0.32 mm i.d. capillary column (GC Care Column BR-Volatile amines fs, Agilent) and nitrogen as carrier gas. The following temperature program was used: starting temperature 40 ºC, heating rate 20 ºC/min and final

temperature 250 ºC. The temperature of the detector was always 250 ºC.

Volatile fatty acids (VFA) and glucose were quantified by high-performance liquid chromatography coupled with a refraction index (HPLC/RI) detector (Varian, Agilent Technologies, Santa Clara, CA, USA) using sulfonated polystyrene resin in the protonated form (67H type) as the stationary phase (Varian Metacarb 67H 300 mm–6.5 mm) and sulfuric acid (0.025 N in milliQ water) as the mobile phase at flow rate of 0.6 mL/min. Column temperature was 65 ºC.

The results reported are the average values from duplicate runs with standard errors lower than 10 %.

3.3. RESULTS AND DISCUSSION

Biodegradability of corrosion inhibitors

Figure 3.1 shows the time course of benzotriazole, quinoline, morpholine and piperazine concentration during the biodegradability test. Owing to the high stability of benzotriazole, it remained unaltered under methanogenic conditions. Hollingsworth et al. (2005) did not observe a significant biodegradation of this compound. However, Liu et al. (2011) reported that benzotriazole can be partially biodegraded when adding iron (III), nitrate and sulfate as electron acceptor, which led to removal efficiencies of 31, 24 and 18 % upon an incubation period of 91 d, respectively. . Later on, Liu et al. (2013) studied the biodegradation of benzotrizole by an inoculum isolated from aquifer sediments. This led to a clear increase of the bentrotriazole removal efficiencies up to 67, 52 and 48 % under iron (III), nitrate and sulfate reducing conditions, respectively, after 77 d. 1–methyl benzotriazole, dimethyl benzylamine and carbazole have been identified as main intermediates products, suggesting methylation, scission of the N-heterocyclic ring and polymerization as possible biotransformation pathways of benzotriazole under anaerobic conditions (Liu et al., 2011; Liu et al., 2013).

Quinoline biodegradation started immediately upon inoculation and lag phase was not observed, which suggest that quinoline is an easily biodegradable compound under anaerobic conditions. Actually, quinoline was completely removed in 13 d when treating initial concentrations lower than 50 mg/L. Nevertheless, increasing concentrations led to a significant reduction of the specific removal rates. The degradation of higher concentrations described an initial rapid degradation phase followed by a slow phase, and a final step where the removal rate increases again. This behaviour was also observed during the imidacloprid biodegradability tests, where the time course of this insecticide concentration followed a biphasic model (Chapter 5). Several theories have been proposed to explain this behavior. First, the occurrence of different steps of the metabolic pathway or rates in the surface and inner parts of the granules (Broznić et al., 2011). Second, the decrease of the active microbial population over time (Sarmah and Close, 2009). However, the extension of TOC removal indicates that quinoline was only mineralized at the lowest concentration (Figure 3.2). Battersby and Wilson (1989) reported complete degradation of quinoline (60 mg/L) after a lag phase of 15 d and 4 weeks of incubation. The increase of the initial quinoline concentration led to a reduction of the TOC removal rate, which indicates that quinoline was transformed into intermediates compounds. The transformation of quinoline under methanogenic conditions has been scarcely studied. However, sulfate reducing and anoxic conditions seem to promote the biodegradation of quinoline following an initial hydroxylation step at position 2 of the pyridine ring to form of 2-hydroxyquinoline (Johansen et al., 1997a; 1997b). Under methanogenic conditions Pereira et al. (1987) observed a series of enzymatic methylations of the accumulated 2-hydroxyquinoline to 1- and 4-methyl-2-hydroxyquinoline, and 1,4-dimethyl-2-hydroxyquinoline.

Morpholine was partially removed after an acclimation period of 6 d regardless the initial concentration treated. The increase of morpholine

concentration from 10 to 500 mg/L led to a significant increase of the specific initial removal rate from 0.13 to 3.50 mg/gVS·d, which may be due to a metabolic activity enhancement at increasing morpholine concentrations (Helbling, 2015). After a certain time, the biodegradation activity decelerated and the removal efficiency decreased down to 54 %, suggesting the occurrence of severe toxic effects over the anaerobic granular sludge. The analysis of TOC revealed that morpholine was almost mineralized under anaerobic conditions. This finding agrees with Pitoi et al. (2011) who have reported that morpholine could be degraded anaerobically by morpholine-degrading mycobacteria. However, several studies have reported on the aerobic degradation of morpholine, which can degraded by *Mycobacterium* sp., *Arthrobacter* sp., mixed activated sludge and *Pseudomonas* sp. (Combourieu et al., 1998; Meister and Wechsler, 1998; Poupin et al., 1998).

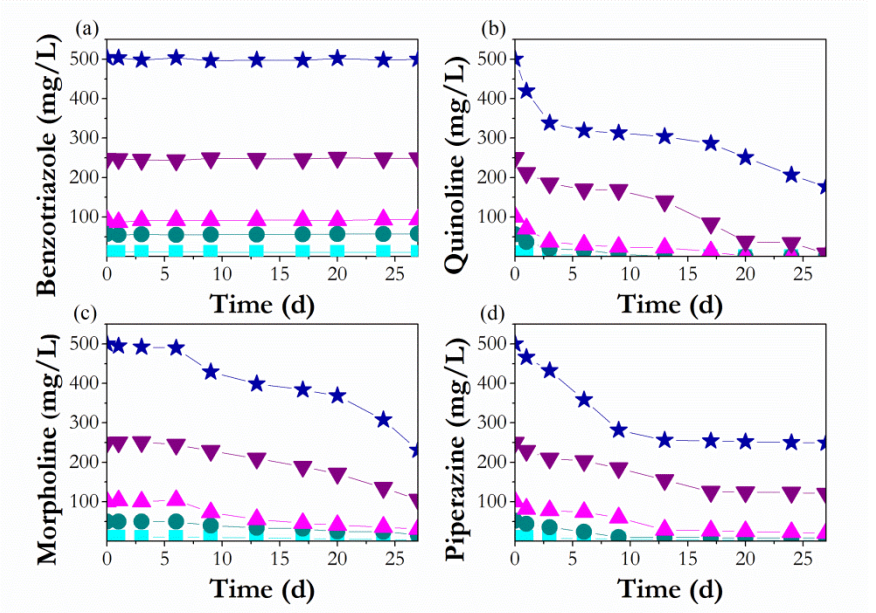


Figure 3.1. Time course of corrosion inhibitors during the biodegradability batch tests for different starting concentrations: 10 (squares), 50 (circles), 100 (pink triangles), 250 (purple triangles) and 500 mg/L (stars) of benzotriazole (a), quinoline (b), morpholine (c) and piperazine (d).

Piperazine was linearly removed at initial stage, then biodegradation activity decelerated and even stopped when treating high concentrations, which could be related to the production of toxic intermediate compounds, as reported by Calza et al. (2008). Thus, although high initial piperazine removal rates were observed at increasing starting concentrations, the removal efficiency frankly decreased to 50 %, which reinforce the hypothesis about the production of undesirable intermediates that could also hinder the TOC consumption (Figure 3.2). Thus, piperazine was transformed into non identified intermediates, which can be toxic and/or inhibitory for the microorganisms responsible for piperazine degradation. Kim et al. (2006) reported that *Mycobacterium* sp. strain THO100, which was isolated from mixed activated sewage sludge was able to utilize morpholine and piperazine as the sole C and N source. Similarly, *Paracoccus* sp. TOH was isolated by Cai et al. (2013), which degraded 100 mg/L of piperazine in 24 h. These works propose the C–N bonds cleavage as a first and second step of the degradation of piperazine to form 2-aminoacetaldehyde which was subsequently transformed into ammonia and glyoxal. Then, glyoxal is converted to oxalic acid, which can be finally mineralized.

Biodegradability of mixtures of corrosion inhibitors

Biodegradability of binary, tertiary and quaternary mixtures of the target corrosion inhibitors was studied. Figure 3.3 shows the initial removal rate of each compound individually and in mixtures. Benzotriazole biodegradation did not suffer any modification in mixtures with other corrosion inhibitors due to its recalcitrant character. Actually, the presence of benzotriazole in binary mixtures led to a decrease of the initial removal rate of quinoline and morpholine. In addition, the low microbial growth yield observed when treating benzotriazole does not support the synthesis of biomass along these experiments (Jia et al., 2006; 2007). In some cases, the initial removal rate of piperazine has been increased when adding benzotrialoze, since the selective pressure driven by its toxicity has led

to microbial consortiums dominated by highly active and toxicity tolerant strains (Jia et al., 2007). This robust species do not compete with more sensitive biomass for piperazine degradation, accelerating the piperazine uptake rate.

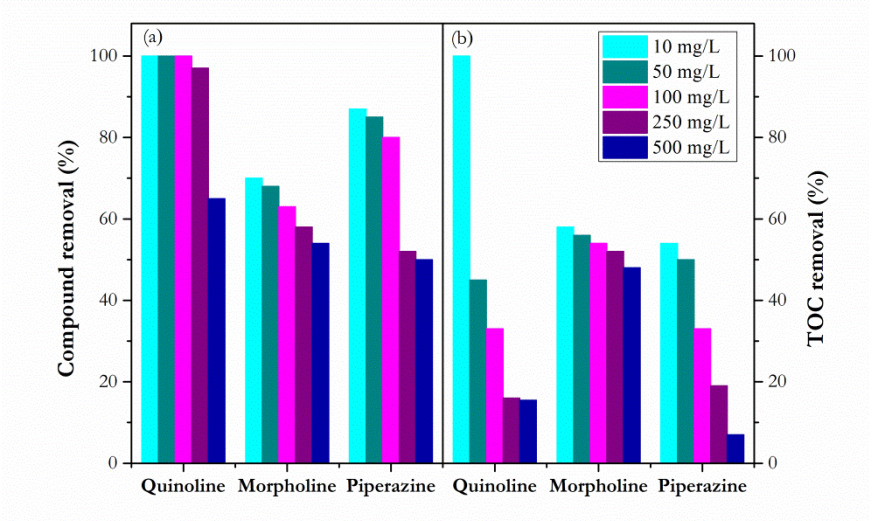


Figure 3.2. Corrosion inhibitor (a) and TOC (b) removal efficiency after 27 d of anaerobic biodegradability test.

The biodegradation of quinoline was strongly affected by the presence of other N-heterocyclic compounds. In binary mixtures the initial removal rate of quinoline was reduced by a 23 and 56 % when benzotriazole and secondary amines were added into the media, respectively. The same behavior was observed in tertiary and quaternary mixtures. The quinoline initial removal rate was reduced between 52 and 64 % in tertiary mixtures, and a 72 % in the quaternary blend. The mechanism of carbon catabolite repression could explain the fact that quinoline elimination was strongly affected by other N-heterocyclic. This mechanism reduce the expression of functions for the use of secondary carbon sources (quinoline) and the activities of the corresponding enzymes in the presence of a preferred carbon source (other N-heterocyclic compounds) (Görke and Stülke,

2008). In contrast, quinoline increased the initial removal rate of other compounds like morpholine, and specially piperazine.

The initial removal rate of morpholine in different mixtures varied from 4 to 9 mg/gVS·d, while its initial removal rate as sole carbon source was 7 mg/gVS·d. The presence of morpholine in binary mixtures containing piperazine induced the activity of the microorganisms responsible of degrading piperazine as reported Schräder et al. (2000). The morpholine removal rate was unlikely affected by the presence of quinoline, which cannot be used as primary carbon source in a cometabolic degradation of the heterocyclic aromatic compounds containing nitrogen.

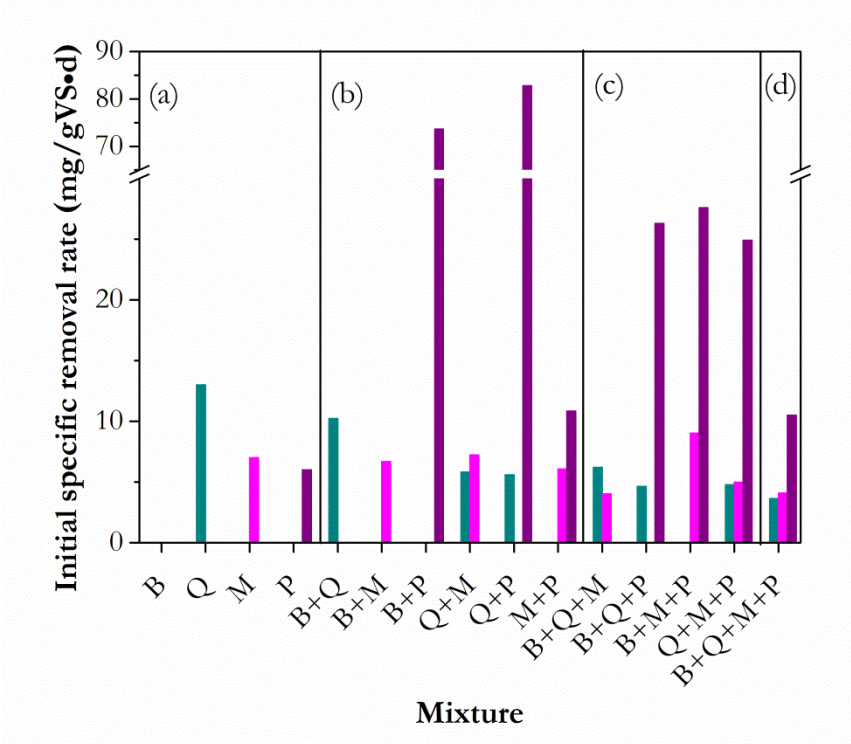


Figure 3.3. Specific initial removal rate for different corrosion inhibitors (B: benzotriazole, Q: quinoline, M: morpholine and P: piperazine): individually (a), binary mixtures (b), tertiary mixtures (c) and quaternary mixture (d).

Finally, the initial removal rate of piperazine was enhanced by the presence of benzotriazole, quinoline and morpholine. This synergistic effect can be attributed to the following mechanisms: first highly toxic compounds like benzotriazole inactivated sensitive microorganisms, allowing the more robust and toxicity tolerant microorganisms to carry out a rapid removal of piperazine. Second, piperazine can be a preferred substrate, which cause the occurrence of the so-called carbon catabolite repression phenomenon and the induction of the enzymes needed for the piperazine degradation. In tertiary mixtures piperazine achieved the lowest initial removal rate when quinoline was present in the blend. Piperazine unlikely affected the degradation rate of quinoline and morpholine, and these compounds improved the removal rate of piperazine, which was identified as apreferred substrate. This finding was in accordance with the results observed in the biodegradability test of the quaternary mixture were the initial specific removal rates followed the next relative order: piperazine>morpholine≈quinoline>benzotriazole.

Methanogenesis inhibition

Inhibitory and toxic effects over the methanogenic activity (acetoclastic and hydrogenotrophic) were evaluated individually. Figure 3.4 describes the normalized acetoclastic (Figure 3.4.a) and hydrogenotrophic (Figure 3.4.b) activity (LCH_4/gVS·d) observed when different concentrations of the target corrosion inhibitors were treated. According to the concentration profiles observed, different inhibition phenomena can be proposed. Thus, irreversible inhibition was observed (toxicity) (Figure 3.5.a) when the biomass did not recover the initial activity adding acetate and formiate as sole carbon source for the acetoclastic and hydrogenotrophic archaea, respectively, or reversible inhibition (Figure 3.5.b) if the granular sludge recovered its activity upon removing the target corrosion inhibitors. In paralell, the inhibition caused on the methane production potential (LCH_4) was evaluated (Figures 3.6 and 3.7).

Benzotriazole concentrations higher than 50 mg/L caused a decrease of the acetoclastic activity of 24 %, which could be caused by the reduction of the growth yield of acetoclastic microorganisms (Jia et al., 2007). Nevertheless, the production of methane remained unaltered, discarding the occurrence of toxic effects. Acetoclastic archaea recovered their activity upon the removal of benzotrizole, which corroborates that benzotriazole is not toxic for acetoclastic methanogens. Hydrogenotrophic archaea were stimulated by benzotriazole concentrations higher than 250 mg/L, which agrees with the stimulating effect over the metabolic activity of some microorganisms reported by Helbling (2015). Nevertheless, the total methane production reached its original value when benzotriazole was removed from the reaction medium. Thus, benzotrizaole was not toxic over the hydrogenotrophics. This finding agrees with previous works where benzotriazole was not toxic to hydrogen-utilizing methanogens (Hollingsworth et al., 2005), as well as acidogenesis and methanogenesis (Tham and Kennedy, 2005).

Increasing quinoline concentrations caused a decrease of the acetoclastic activity, reducing the initial activity by a 65 % when the treating 500 mg/L. In addition, the specific activity of acetoclastic methanogens was not recovered after removing quinoline, proving the toxic character of quinoline over these archaea. Similarly, methane production experienced a reduction up to 46 %. Hydrogenophic activity showed more robust against quinoline and an increasing activity was even observed when adding this species. Recovering the original removal and methane production activity after removing quinoline. In this study the concentration that caused a 50 % of inhibition over acetoclastic archaea was between 250 and 500 mg/L. Johansen et al. (1997b) obtained an EC_{50} of 50 mg/L under desnitrifying conditions. *D. indolicum* tolerates quinoline up to 64 mg/L without any inhibitory effects under sulfate-reducing conditions (Licht et al., 1997).

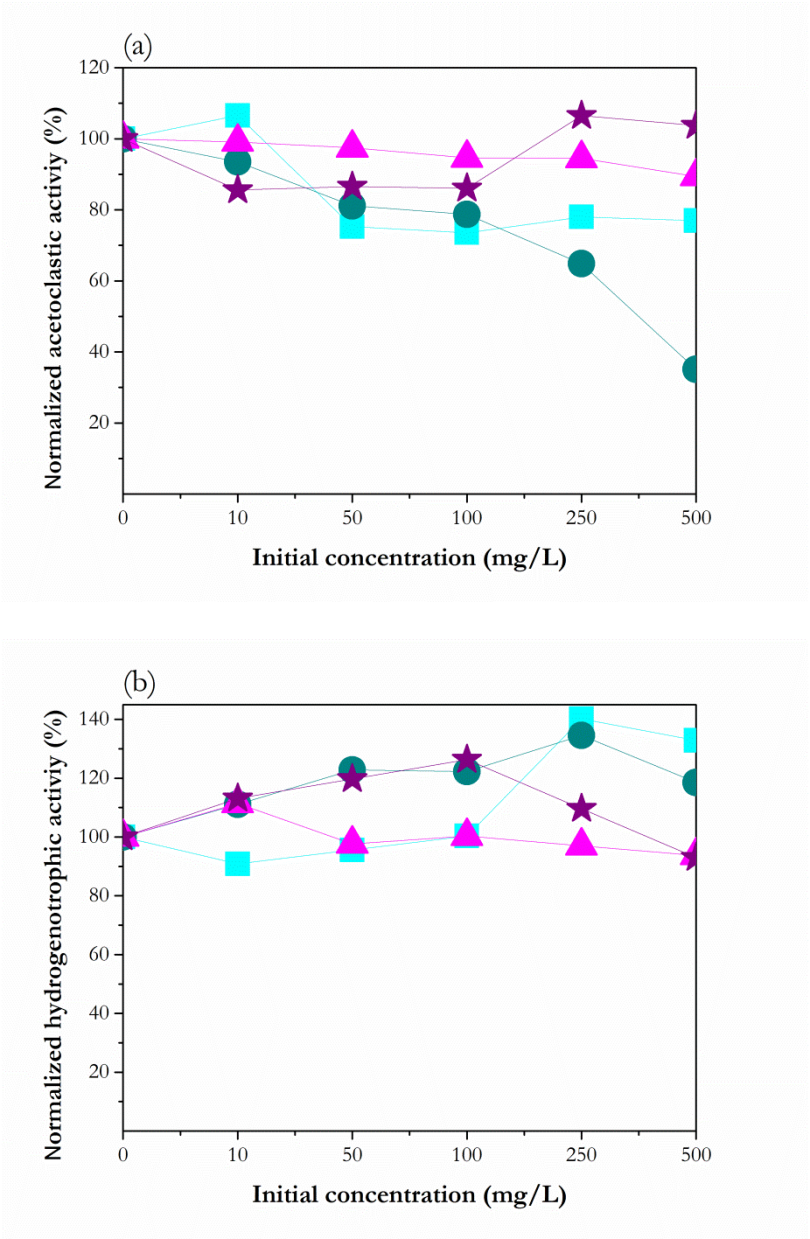
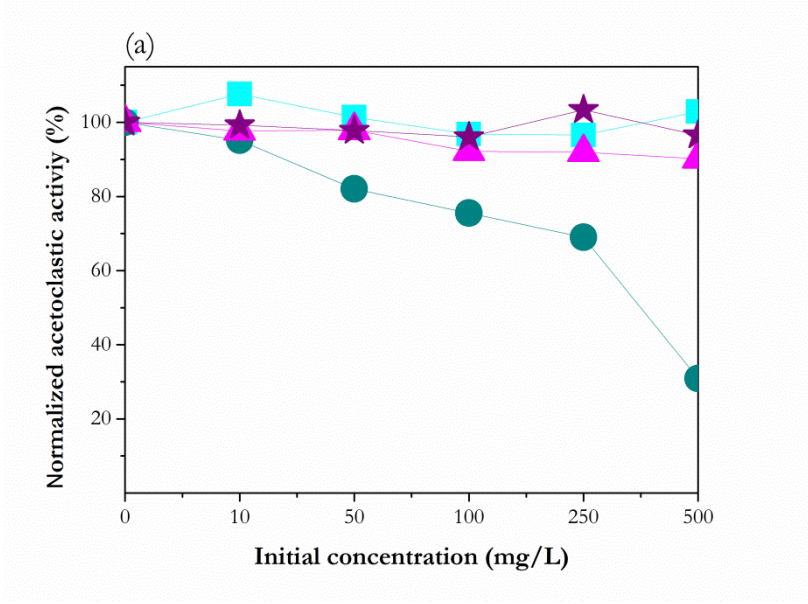


Figure 3.4. Normalized acetoclastic (a) and hydrogenotrophic (b) activity in presence of different corrosion inhibitors (benzotriazole (squares), quinoline (circles), morpholine (triangles) and piperazine (stars)) at different starting concentrations during the inhibition assays.



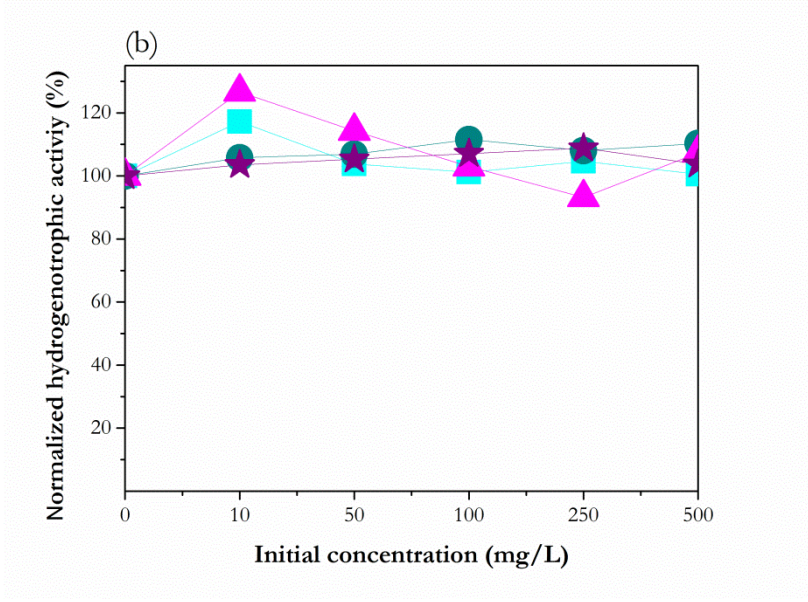


Figure 3.5. Normalized acetoclastic (a) and hydrogenotrophic (b) activity during the recovery assay (benzotriazole (squares), quinoline (circles), morpholine (triangles) and piperazine (stars)) when the sole carbon source was acetate for the acetoclastics and formiate for hydrogenotrophics.

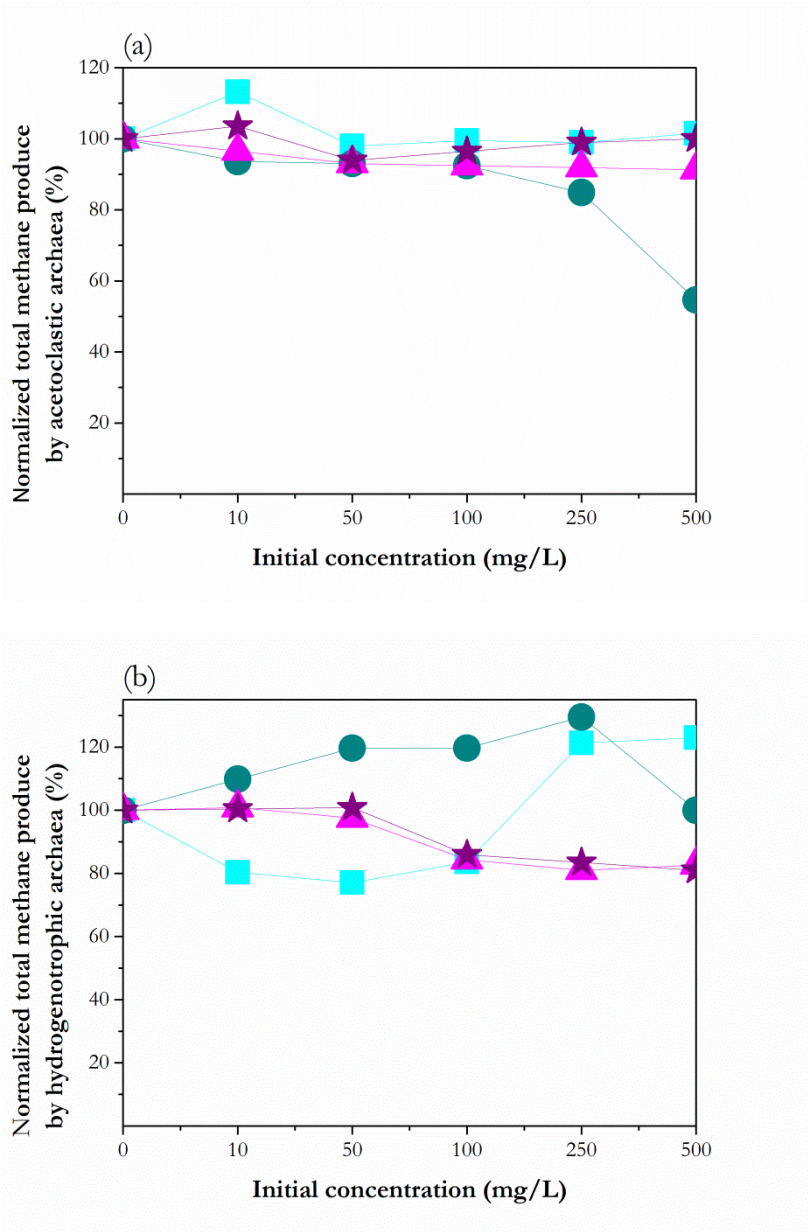


Figure 3.6. Normalized total methane produce by acetoclastic (a) and hydrogenotrophic (b) archaea in presence of different corrosion inhibitors (benzotriazole (squares), quinoline (circles), morpholine (triangles) and piperazine (stars)) at different starting concentrations during the inhibition test.

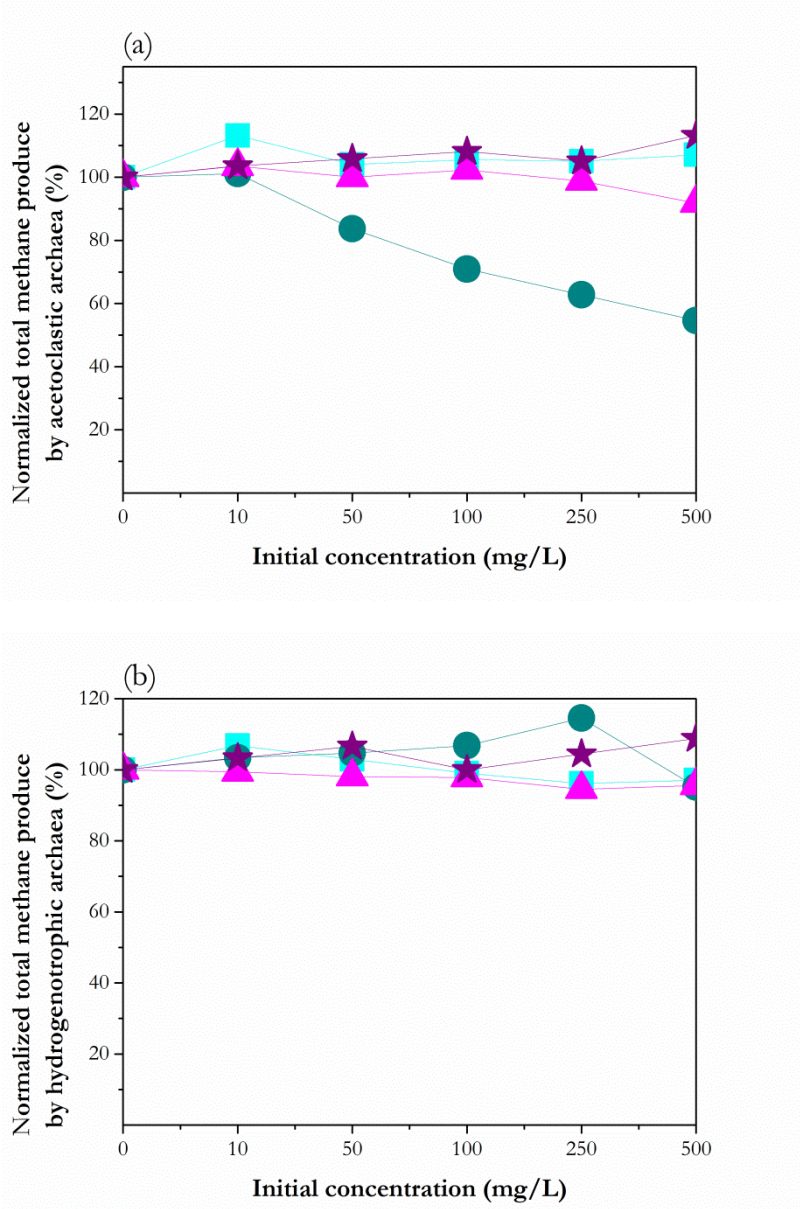


Figure 3.7. Normalized total methane produced by acetoclastic (c) and hydrogenotrophic (d) biomass during the recovery assay (benzotriazole (squares), quinoline (circles), morpholine (triangles) and piperazine (stars)) when the sole carbon source was acetate for the acetoclastics and formiate for hydrogenotrophics.

A slight reduction of 10 % of the acetoclastic activity and total methane production was achieved when 500 mg/L of morpholine was treated. This activity reduction was permanent, suggesting that morpholine is slightly toxic for the acetoclastic archaea. This effect was even more moderate over the hydrogenotrophic archaea activity (7 %) and methane production (22 %), which was recovered once morpholine was removed.

Piperazine did not cause inhibitory or toxic effects over the acetoclastic and hydrogenotrophic archaea. Even, this N-heterocyclic compound stimulated the hydrogenotrophic activity reaching a maximum when treating an initial concentration of 100 mg/L. This secondary amine decreased the total methane production of hydrogenotrophic archaea about 20 % when adding initial concentrations higher than 100 mg/L, which was recovered in the absence of piperazine.

From the best of our knowledge, there is no information available about the inhibitory/toxic effect of morpholine and piperazine over methanogenesis, being evaluated under aerobic conditions. Knapp et al. (1982) did not observe any toxic effects of morpholine on bacteria *Mycobacterium* sp. MorG, in concentrations up to 8.7 g/L. Mazure and Truffaut (1994) concluded from their experiments that the toxicity threshold of morpholine would lie between 5 and 7 g/L for *Mycobacterium aurum* MO1.

Jia et al. (2007) described several mechanisms which might be involved in the inhibitory effects of toxic compounds on methanogenic microbial activity. They suggest that toxicants could reduce the substrate uptake of the active organisms, may kill a fraction of the microbial population, and reduce the growth yield.

As in next Chapters (5, 6 and 7) hydrogenotrophic biomass was more resistant than the acetoclastic ones. This behavior could be attributed to the inhibition of complex and coenzymes related with methane production from acetate and the lack of a protective envelope around

the cell wall (Chidthaisong and Conrad, 2000; Gerardi, 2003; Smith and Ingram-Smith, 2007). In general, the inhibition and toxic effect over the removal activity was higher than that observed over the methane production. The reasons for this difference (discussed in Chapter 5) were: i) a decrease in degradation rate (Strek, 1998), ii) a kinetic impact, due to it would take more time for the completion of microbial activity ending with full utilization of available organic substrate and therefore producing the same amount of methane in the absence of inhibitory/toxic compound (Cetecioglu et al., 2012) and iii) microorganism populations may change over time (Sarmah and Close, 2009).

Long-term continuous experiment

The EGSB reactor was operated for 157 d (Figure 3.8), being necessary a start-up period of around 35 d for the system stabilization. Once the reactor reached the steady state, corrosion inhibitors were spiked into the feed. The addition of N-heterocyclic compounds caused a drastically drop in the COD removal and methane production efficiency. The COD removal efficiency was recovered linearly until day 143, which remained constant around 80 %. Methane production was also recovered, achieving an efficiency of 0.38 gCH_4-COD/gCOD. However, it not reached its initial efficiency, suggesting a certain inhibitory phenomenon related with a slower microbial growth yield (Jia et al., 2006; 2007), a decline in microbial viability (Strek, 1998) and a decrease of the microorganism population over time (Sarmah and Close, 2009) due to the inhibitory/toxic effects showed by these N-heterocyclic compounds over methanogens in previous experiments.

As in the biodegradability tests, benzotriazole concentration remained constant along the continuous experiment. No evidence for anaerobic degradation of benzotriazole also found by Hollingsworth et al., (2005). Even, when an easy biodegradable carbon source was added (ethylene glycol) benzotriazole remained unaltered but did not impact

on the ethylene glycol consumption (Tham and Kennedy, 2005). This finding is in accordance with the results reported by Herzog et al., (2014) who observed that nitrogen availability is more important than carbon supply for benzotriazole degradation.

Quinoline biodegradation described a slow initial degradation stage (0.29 mg/L·d) during the first 73 d. After that, a fast stage was observed, in which quinoline degradation rate was 16-fold times higher (4.62 mg/L·d). At this point (day 118) quinoline was almost depleted. The start of the rapid phase of quinoline degradation occurred when piperazine was degraded around 90 %. This finding is in accordance with the result achieved in biodegradability test where quinoline degradation was strongly affected by secondary amines while piperazine degradation was enhanced by adding quinoline.

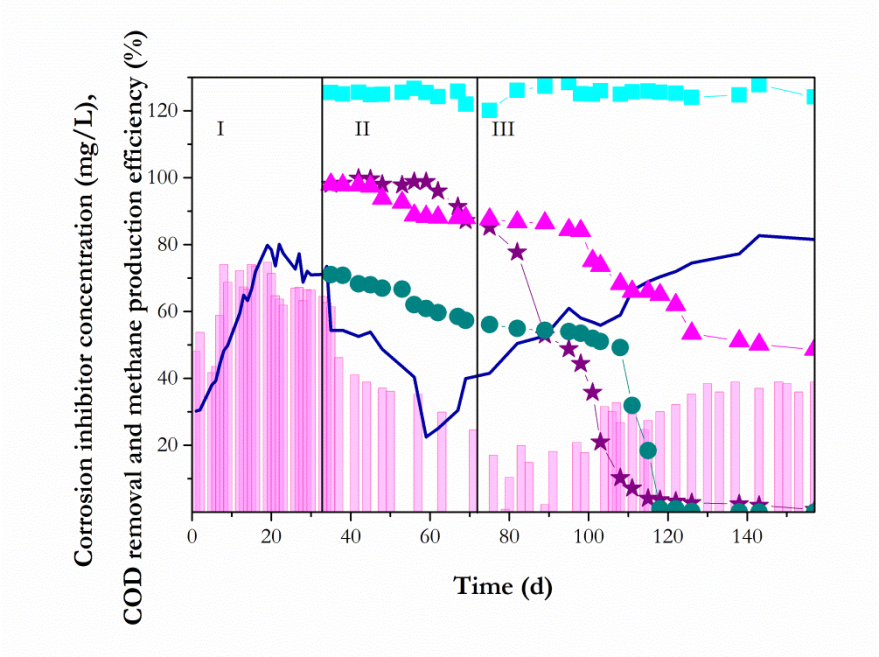


Figure 3.8. Time course of the COD removal efficiency (line), methane production (bars), and concentration of benzotriazole (squares), quinoline (circles), morpholine (triangles) and piperazine (stars) detected in the effluents discharged from EGSB reactor.

Morpholine degradation started after an acclimation period of 10 d. Then, morpholine concentration decreased by 12 % (day 59), when piperazine biodegradation started and the morpholine degradation rate decreased and almost stopped. When piperazine removal reached a value of 55 %, morpholine elimination was resumed until day 108, achieving a removal efficiency of 66 %. The same day piperazine was almost eliminated and the rapid quinoline removal phase was initiated, which stopped the morpholine biodegradation, being re-started once quinoline was consumed (day 118). This finding agrees with the assays of biodegradability mixtures in which the preferred carbon source was piperazine and also highlights that the following preferred substrate was quinoline.

Piperazine removal needed an acclimation phase of 24 d. After this period, a removal efficiency of 97 % was achieved. The piperazine removal was linked to morpholine uptake, since enzymes responsible for piperazine degradation are induced during the morpholine removal (Knapp et al., 1982). This step took 14 d after which piperazine degradation began and the mechanism of carbon catabolic repression took place. During this phenomenon piperazine was the preferred carbon source and the activities of the enzymes responsible for the degradation of morpholine were reduced.

Volatile fatty acids and glucose were added into the feed during the long-term experiment as growth substrates. The concentration of acetate, propionate, butyrate and glucose is shown in Figure 3.9. Acetate and propionate were almost undetectable in the effluent, and only low concentrations of butyrate were detected in the effluent. Therefore, VFA degradation seemed unaffected by N-heterocyclic compounds. Once the N-heterocyclic compound were fed, glucose concentration suffered a drastically increase, but its degradation was efficiently recovered in 21 d. Owing to the production of intermediates from the N-heterocyclic removal, glucose elimination was negatively affected. Nevertheless, when quinoline started it fast

degradation stage (day 108), glucose consumption dropped, suggesting that glucose biodegradation could be affected by the degradation of quinoline, indicating an inhibition of the fermentative microorganisms, which was subsequently recovered. The same result was observed by Jianlong et al. (2002) who studied the kinetics of co-metabolism of quinoline and glucose by *Burkholderia pickettii*. Puyol et al. (2009) also reported a significant decrease of glucose consumption rate in presence of 2,4-dichlorophenol (2,4-DCP) but fermentative microorganisms were not completely inhibited despite the toxic character of 2,4-DCP.

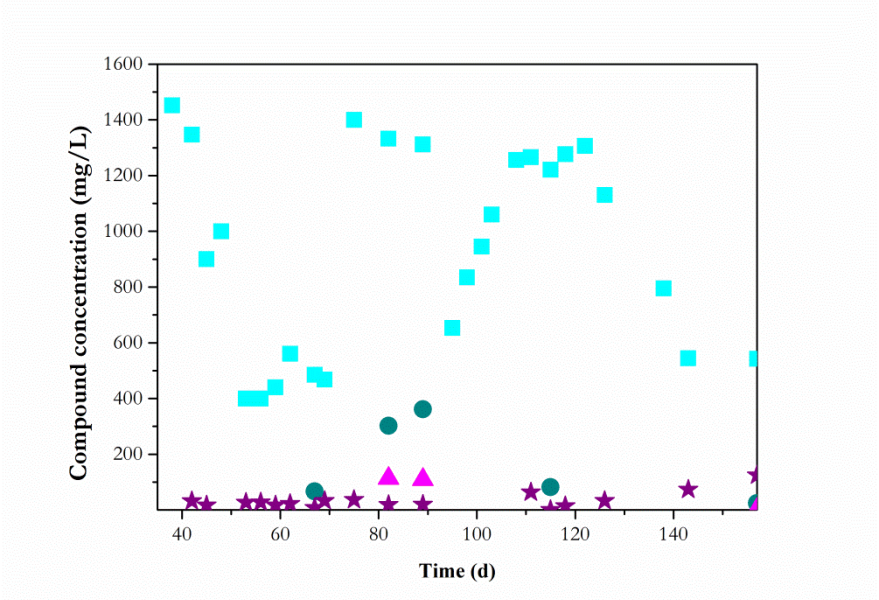


Figure 3.9. Glucose (squares), acetate (circles), propionate (triangles) and butyrate (stars) concentration in the effluent during the EGSB operation treating a corrosion inhibitors bearing wastewater.

Sequencing analysis

Archaea and bacteria community were analyzed by means of Illumina sequencing technology before and after the treatment of a wastewater containing corrosion inhibitors. Figures 3.10 and 3.11 show the relative abundance of archaea at specie level using different primers. Although both primers can amplify both archaea and bacteria, the

results obtained cannot be directly compared to each other because they have different biases and amplify different 16S regions. Nevertheless, similar trends were found. In both assays genus *Methanobacterium* was identified. *Methanobacterium* strains play an important role in the anaerobic degradation of organic compounds as the terminal metabolic groups (Hobson and Shaw, 1973; Ma et al., 2005). When the primer for archaea was used, the relative abundance of the genus *Methanobacterium* increased during the treatment, but the number of operational taxonomic units (OTUs) drastically decreases from 2070 to 168. The results obtained when the primers for bacteria were used revealed a clear reduction in the relative abundance of the genus *Methanobacterium*, as well as a decrease in the OTUs from 888 to 34. These results show that *Methanobacterium* genus have a stronger survival and tolerance capacities than previously reported in previous works (Lin et al., 2013). Within this genus can be highlighted the presence of *Methanobacterium beijingense*, *Methanobacterium* sp. and an unknown specie of *Methanobacterium*. The most resistant was *Methanobacterium beijingense*, a hydrogenotrophic archaea (Ma et al., 2005), due to it only reduce its OTUs by a 61 and 81 in archaea and bacteria assay, respectively. The rest of species decreased by more than 93 %.

Methanosaeta genus was identified in the bacteria assays. This acetoclastic group and the hydrogen/formate-scavenging *Methanobacterium* group are the two main mesophilic groups commonly found in granular sludge of anaerobic methanogenic bioreactors (Celis et al., 2009). At the beginning of the treatment *Methanosaeta* genus represented a 58 % (1245 OTUs) of the archaea population. The seed sludge was collected from a UASB reactor which favors the presence of *Methanosaeta* sp. (Leclerc et al., 2004). At the end of the experiment only 557 OTUs belong to *Methanosaeta* genus likely because *Methanosarcinaceae* were sensitive to the turbulence and shear associated with high-efficiency reactors (Kobayashi et al., 1988) in addition to a possible inhibition caused by the N-heterocyclic compounds.

Methanosaeta concilii represented a 57 % of the archaea in the initial granular sludge identified by the bacteria primer. The presence of *Methanosaeta concilii* is an indicator of a high rate substrate conversion to methane gas by methanogens (Ambuchi et al., 2016). However, at the end of the treatment this specie reduced its abundance to 13 %, which was associated to the reduction in the methane production. Nevertheless, unknown specie belongs to *Methanosaeta* genus appeared during the treatment representing an 81 % of the archaea community. According with this finding, *Methanobacterium* genus was replaced by *Methanosaeta*, this behavior was also reported by Lin et al., (2013).

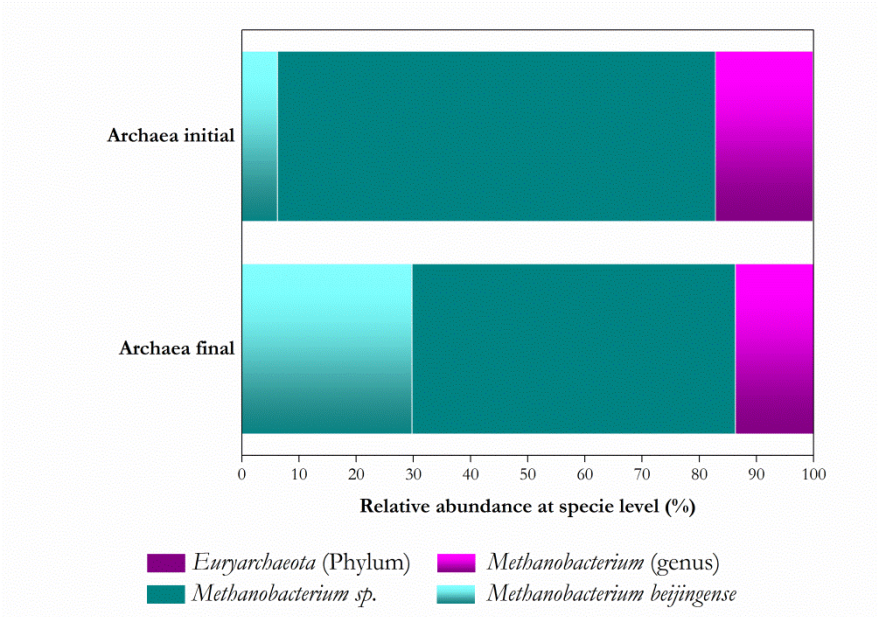


Figure 3.10. Achaea diversity at specie level obtained using the primers for archaea 519wF-909R.

Methasarcina mazei and *Methanomethylovorans* were detected in the seed sludge. *M. mazei* is frequently observed in anaerobic digestion systems and is able to utilize a variety of substrates, including acetate, H_2/CO_2, methanol and methyl-amines (Bialek et al., 2011). The genus *Methanomethylovorans* can utilize methanol, methylated amines, dimethyl sulfide and methanethiol (Lomans et al., 1999). *Methanomethylovorans*

strains have also been detected in samples from rice-field soils, oil-contaminated groundwater, freshwater sediments, sludge from an anaerobic baffled reactor treating industrial dye waste and in a bioreactor treating dichloropropane-contaminated wastewater (Jiang et al., 2005).

Joshi et al. (2016) have identified the genus *Methanosarcina*, *Methanobacterium*, *Methanomethylovorans* and *Methanosaeta* during the treatment of coking wastewater containing N-heterocyclic compounds in a long-term sequential anaerobic-aerobic bioreactor.

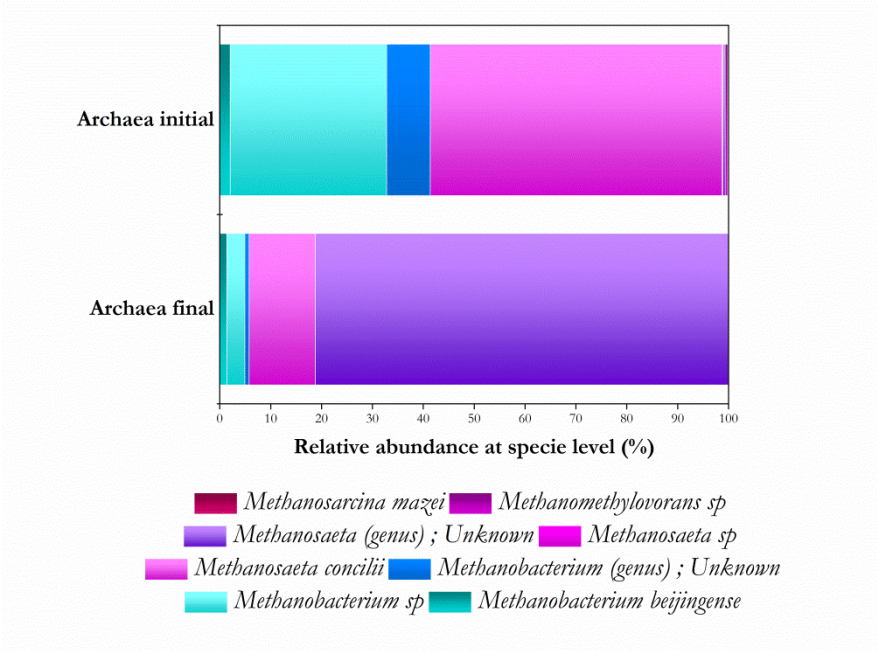


Figure 3.11. Achaea diversity at specie level obtained using the primers for bacteria 357wF-785R.

The relative abundance of bacteria at specie level is depicted in Figure 3.12. A clear shift in bacteria community took place during the treatment of wastewater bearing corrosion inhibitors. In the seed sludge the predominance phylum was *Firmicutes* (41 %), followed by *Bacteroidetes* (10 %), *Proteobacteria* (8 %) and *Synergistetes* (8 %). However,

at the end of the treatment, genus *Proteobacteria* (65 %) prevailed over *Firmicurtes* (18 %) and *Synergistes* (3 %). Bae et al. (2009) reported that heterocyclic amine-degrading denitrifiers are widely distributed in the *Proteobacteria* groups.

Firmicutes are syntrophic bacteria, which can degrade VFA such as butyrate and its analogs. This degradation produces H_2, which is then degraded by hydrogenotrophic methanogens (Riviére et al., 2009). Within this phylum the genus *Syntrophomonas* was identified in the seed sludge and remained until the end of the long-term experiment. These syntrophic acetogenic bacteria have an important role in fatty-acids degradation and work in a synergetic way together with hydrogen-scavenging microbes such as hydrogenotrophic methanogens (Merlino et al., 2012). At the beginning of the treatment bacteria belonged to *Clostridia* class were detected. *Clostridia* are strict anaerobes which are involved in the stages of hydrolysis, acidogenesis, acetogenesis and syntrophic acetate oxidation (Ziganshin et al., 2013). *Firmicutes Erysipelotrichaceae* was also identified in the inoculum, which is involved in acidogenesis. Some species ferment glucose, and major metabolic end-products are butyrate, lactate and formate (Si et al., 2016).

Bacteroidetes, known to be proteolytic bacteria, intervene in the degradation of proteins and are able to ferment amino acids to acetate (Riviére et al., 2009). Members of the *Bacteroidetes* are also commonly detected in anaerobic sewage sludge digesters, in biogas reactors fed with plant biomass and in digesters fed with protein-rich biomass (Ziganshin et al., 2013). Only *Petrimonas* sp. has been detected among this phylum. Strains in *Petrimonas* ferment sugars with acetate and propionate as major fermentation products and/or a small amount of succinate (Guo, et al., 2014), and also it has been shown to produce H_2 (Lakaniemi et al., 2011).

Members of the *Proteobacteria* group are widely distributed in nature and play an important role in the mineralization of organic matter (Lin et al., 2012). Inside the phylum *Proteobacteria*, members belonged to the classes *Beta*, *Delta* and *Gammaproteobacteria* were detected.

Betaproteobacteria are the main consumers of propionate, butyrate and acetate (Riviére et al., 2009). Within this class *Alcaligenes* sp. was identified in the seed sludge. *Alcaligenes* sp. is able to degrade a wide spectrum of organic compounds (Rajeshkumar and Jayachandran, 2003). *Syntrophobacter* genus which belonged to *Deltaproteobacteria* class was detected before the addition of corrosion inhibitors into the feed. This genus is syntrophic propionate-degrading bacteria (Liu et al., 1999). *Syntrophobacter* sp. transforms propionate to acetate, H_2 and CO_2, but only when co-cultivated with H_2-consuming organisms (Lakaniemi et al., 2011). Inside the class *Gammaproteobacteria*, *Idiomarina* genus, *Pseudoalteromonas* sp., *Alcanivorax* sp., *Halomonas* sp. and *Pseudomonas* sp. were identified at the end of the treatment. *Idiomarina* genus is known as a versatile heterotrophs (van der Kraan et al., 2009), belonging to *Idiomarinaceae* family. This includes facultative anaerobic microorganisms (Ivanova et al., 2004) as *I. loihiensis* which inhabits partially oxygenated cold waters (Hou et al., 2004). *Pseudoalteromonas* strains pose a high hydrolytic activity, especially in the degradation of a huge range of polysaccharides (Ivanova et al., 1998). Genus *Alcanivorax* was found in oil-contaminated marine environment, especially when nitrogen and phosphorus fertilizers are added to stimulate the growth of endogenous microorganisms (Harayama et al., 1999). *Alcanivorax* species have the ability to degrade polycyclic aromatic hydrocarbons (PAHs) and alkanes (Brito et al., 2006). Genus *Halomonas* has been reported to cleave aromatic rings (Melcher et al., 2002). *Pseudomonas* strains are capable of degrading recalcitrant compounds (Pant and Adholeya, 2007). Zhang et al. (2011) concluded that *Pseudomonas* can be considered as a quinoline-degrading bacterial community.

Synergisteteses phylum can use amino acids and in turn provide short-chain fatty acids and sulphate for terminal degraders such as the methanogens and sulphate-reducing bacteria (Riviére et al., 2009). Among this phylum, the order *Synergistales* was found before and after the treatment of corrosion inhibitors bearing wastewater. Before the

addition of N-heterocyclic compounds into the feed *Aminivibrio pyruvatiphilus* and *Synergistes* sp. were identified. However, after the treatment only the latter was detected. *Aminivibrio pyruvatiphilus* could ferment amino acids and organic acids (Honda et al., 2013), while the proteolytic anaerobic bacterium *Synergistes* sp. is characterized by its hydrolytic enzyme (Kumar et al., 2008).

3.4. CONCLUSIONS

N-heterocyclic compounds with 2 or less nitrogen atoms were biodegraded under anaerobic conditions in batch test experiments while compounds with 3 nitrogen atoms were not removed within 27 d of biodegradability test. Quinoline degradation was severely affected by the presence of other corrosion inhibitors while piperazine degradation was enhancement due to piperazine was the preferred carbon source. The higher the number of components in the mixture the less initial removal rate for each compound. Corrosion inhibitors were not toxic for hydrogenotrophic biomass while quinoline caused severe toxic effects over acetoclastic archaea. A long period was required to achieve the steady state in presence of N-heterocyclic compounds, but finally quinoline and piperazine were successfully removed from wastewaters in an EGSB reactor. A clear shift occurred in the microbial diversity being *Methanosaeta* genus and *Proteobacteria* phylum the dominant archaea and bacteria at the end of the treatment, respectively.

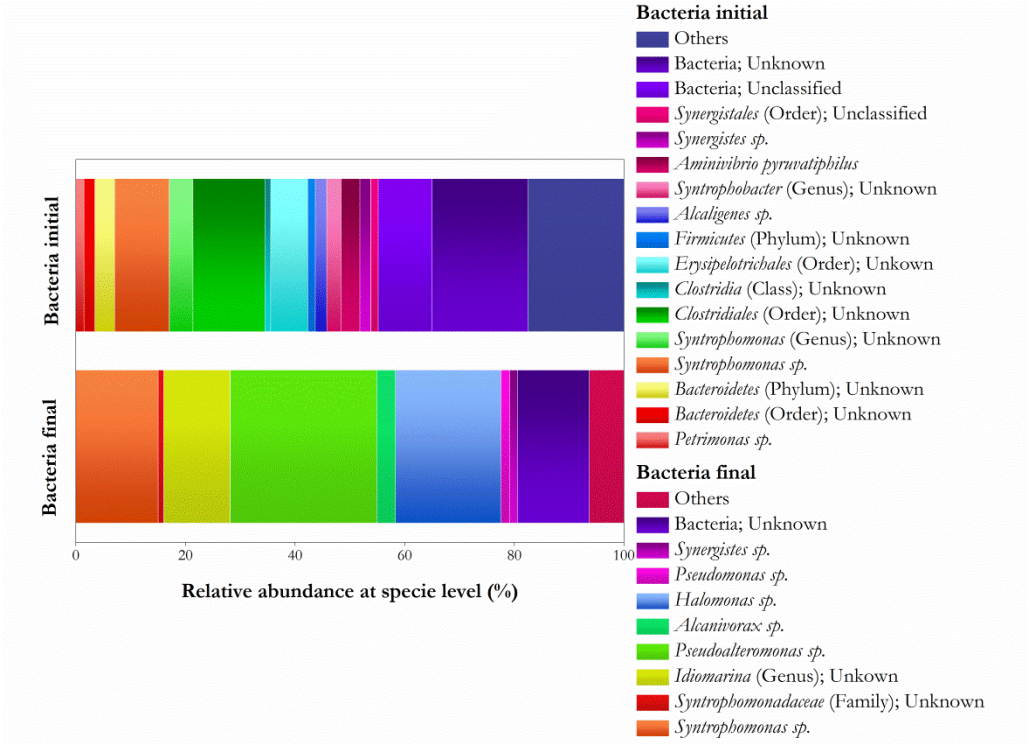


Figure 3.12. Bacteria diversity at specie level obtained using the primers for bacteria 357wF-785R. Species making up less than 1 % were defined as "Others".

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4

ANAEROBIC BIODEGRADABILITY OF MIXTURES OF PESTICIDES IN AN EXPANDED GRANULAR SLUDGE BED REACTOR

Monsalvo, V. M., Garcia-Mancha, N., Puyol, D., Mohedano, A. F., & Rodriguez, J. J. (2014). Anaerobic biodegradability of mixtures of pesticides in an expanded granular sludge bed reactor. *Water Science and Technology*, *69*(3), 532-538.

4. ANAEROBIC BIODEGRADABILITY OF MIXTURES OF PESTICIDES IN AN EXPANDED GRANULAR SLUDGE BED REACTOR

Abstract

The biodegradability and toxicity of three commercial pesticides containing 2-methyl-4-chlorophenoxyacetic acid (MCPA), imidacloprid and dimethoate were evaluated individually, and a complex mixture of these pesticides was treated in an expanded granular sludge bed (EGSB) reactor. MCPA was partially biodegraded, while imidacloprid and dimethoate remained almost unaltered during the individual biodegradability tests. Cyclohexanone was identified as the major solvent in the dimethoate-bearing insecticide, which was completely removed regardless of the presence of other pesticides. The analysis of the inhibition over the acetoclastic methanogenesis showed IC_{50} (half maximal inhibitory concentration) values of 474 and 367 mg/L for imidacloprid and dimethoate, respectively. The effect on the methanogenesis was negligible in the case of MCPA and cyclohexanone. Pesticides caused a dramatic decrease of the EGSB reactor performance. After 30 d acclimation, the EGSB reactor achieved a stable chemical oxygen demand (COD) removal efficiency and methane production of around 85 % and 0.9 gCH_4-COD/gCOD, respectively, for MCPA, imidacloprid, dimethoate and cyclohexanone feed concentrations of 57, 20, 25 and 27 mg/L, respectively. The presence of complex pesticide mixtures led to synergistic/antagonistic responses, reducing the MCPA biodegradation and improving the removal of the insecticides' active ingredients, which were completely removed in the EGSB reactor.

4.1. INTRODUCTION

Although the benefits generated by the application of agrochemicals are evident in terms of increased agricultural productivity and improved public health through disease control, the presence of pesticide residues in the environment has created a potential risks perspective. Herbicides and insecticides are chemically stable and recalcitrant, and represent a risk to health and the environment, due to their persistent and long-term toxicity (Sarkar et al., 2008). During the last decade, one of the most commonly used selective herbicides in agricultural areas is 2-methyl-4-chlorophenoxyacetic acid (MCPA). Imidacloprid is at the moment the insecticide with the world's fastest growing sales and is considered a possible replacement for the widely used organophosphate insecticides like dimethoate, which is subject to phased revocation in many countries. The intensification of agriculture has led consequently to an increasing demand of these pesticides, which have been found in wastewaters and water reservoirs (Ballesteros Martín et al., 2009). For these reasons, some pesticides are considered as priority pollutants (Directive 2000/60/EC) and the use of these three active ingredients, MCPA, imidacloprid and dimethoate, is regulated by the European Directives 2007/27/EC, 2006/626/EC and 2002/71/EC.

The main causes of water pollution by pesticides include the use of pesticides as a routine practice in agriculture and their subsequent presence in surface run-off, the cleaning of recipient and dispensing equipments, the preparation of agricultural products such as fruit and vegetable washing, and disposal of polluted plants (Devipriya and Yesodharan, 2005). To prevent the impact of these compounds it is necessary to develop methods allowing their effective breakdown. The application of biological processes is considered a promising cost-effective and environmentally sustainable alternative for the treatment of such wastewaters. Although conventional biological treatment systems can be seriously affected by the presence of pesticides in wastewaters (Ballesteros Martín et al., 2010), aerobic advanced

biological systems have the potential to remove toxic compounds from wastewater (Sanchis et al., 2013). Against the high relative operating costs of these systems, anaerobic bioreactors are considered as a promising effective technique able to remove these pollutants (Mikesell and Boyd, 1985; Ghosh et al., 2001). However, there is limited information on the feasibility of anaerobic bioreactors for this purpose so far. The fluidized bed reactor, membrane bioreactor and sequencing batch reactor operating under anaerobic conditions have been claimed as effective technologies for the removal of isoproturon and 2,4-dicholophenoxyacetic acid (Buenronstro-Zagal et al., 2000; Chin et al., 2005; Celis et al., 2008), but their operational complexity and associated costs limit their application.

Among the anaerobic systems, the upflow anaerobic sludge blanket reactor is the most widely applied for industrial wastewater treatment. However, the so-called expanded granular sludge bed (EGSB) reactor is a promising alternative where the height to diameter ratio and the external recirculation rate are increased, improving the mixing and contact between wastewater and biomass. EGSB technology has been reported to be adequate for dealing with hardly biodegradable wastewater, and to dampen the inhibition of the microbial activity caused by the presence of hazardous pollutants (Tauseef et al., 2013). Thus, the viability of this system has been previously demonstrated in full-scale applications treating real wastewaters (Seghezzo et al., 1998).

The aim of this work is to evaluate the anaerobic biodegradability of a commercial selective herbicide, a neonicotinoid insecticide and an organophosphate insecticide, whose active components are MCPA, imidacloprid and dimethoate, respectively. Cyclohexanone was also studied due to its high concentration in the dimethoate-bearing commercial insecticide. Batch experiments were used for evaluating the viability of the anaerobic process, whereas long-term experiments through an EGSB reactor were conducted for studying the treatability of low-strength wastewater containing these pesticides.

4.2. MATERIALS AND METHODS

Chemicals

A commercial selective herbicide (60 % w/v MCPA) was purchased from Fertiberia (Spain). The neonicotinoid insecticide was employed as Couraze® (20 % w/v imidacloprid) and the organophosphate insecticide as Danadim® (40 % w/v dimethoate). Both insecticides were supplied by Cheminova Agro, S.A. (Spain) and the last one contains cyclohexanone as the main solvent (43 % w/v). Other chemicals used in these experiments were of reagent grade and used as received.

Wastewater composition

Synthetic wastewater was prepared by adding the following components (mg/L): peptone (17.4), yeast extract (52.2), milk powder (116.2), sunflower oil (29.0), sodium acetate (79.4), starch (122.0) and urea (91.7) to get a total chemical oxygen demand (COD) of around 1.75 g/L. The fed wastewater was supplemented with 20 mL/L of the following micronutrient solution (µg/L): $FeCl_2 \cdot H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO \cdot 5H_2O$ (162), EDTA (1,000), resazurin (0.2), sulphuric acid 36 % (1 µL/L). Alkalinity was provided by adding 1 $gNaHCO_3/gCOD$. Pesticides were incorporated as a fraction of total organic carbon (TOC) of 20 %.

Biodegradability and methanogenesis inhibition tests

Biodegradability tests were performed for 21 d inoculating 1.5 g volatile solids (VS)/L of non-adapted granular sludge by adding each pesticide individually to reach different concentrations of the active ingredients (10–500 mg/L) to a standard methanogenic medium containing the following macronutrients (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328), $MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and yeast extract (4). This medium was supplemented with 1

mL/L of the aforementioned micronutrients solution and $NaHCO_3$ as previously described. The biodegradability test was performed at 30±1 ºC in duplicate, using the Automatic Methane Potential Test System (AMPTS, Bioprocess Control, Sweden) described elsewhere (Garcia-Mancha et al., 2012). The inhibition of acetoclastic methanogenesis was studied by activating the anaerobic sludge with sodium acetate (4 g/L), according to García-Mancha et al. (2012). Contribution of adsorption was evaluated in biomass samples after extraction with Soxhelt following the US EPA 8041 method (EPA, 1995). Tests of volatilization were performed under identical operating conditions as in the biodegradation experiments but in absence of biomass.

EGSB reactor description and operation

Experiments in continuous mode were carried out using a 5.2 L EGSB reactor with an internal diameter to height ratio of 1:7.2. The reactor was equipped with a gas–liquid– solid separator installed 15 cm below the exit. The wastewater was continuously fed entering at the bottom of the reactor with the recirculation, and the effluent was withdrawn from the top. CO_2 was removed from biogas using a Mariotte flask with 4 M NaOH solution trap, and CH_4 was measured with a wet gas meter (Schlumberger, Germany). The reactor was operated at an upward flow rate of 2.5 m/h and 35 ºC for 90 d. The EGSB reactor was inoculated with 100 g VS of granular sludge previously activated adding the synthetic wastewater without pesticides. Macro- and micronutrients, as well as $NaHCO_3$ (buffer and alkalinity source) to neutralize the influent, were supplemented as above indicated. The organic loading rate (OLR) and the hydraulic retention time were maintained at 1.75 gCOD/L·d and 1 d, respectively. The concentration of pesticides in the feed was kept constant along the experiment at 57, 20, 25 and 27 mg/L for MCPA, imidacloprid, dimethoate and cyclohexanone, respectively.

Analytical methods

Analyses of COD, total solids and VS were performed according to APHA Standard Methods (APHA, 1992). TOC was measured by a

Shimadzu TOC-$V_{CPH/CPN}$ analyzer. MCPA was quantified by high-performance liquid chromatography (HPLC) (Varian Prostar 325) with a UV detector and a Teknokroma Mediterranea Sea-18 column (25 cm length, 4.6 mm i.d.) as the stationary phase. Analyses of MCPA were carried out at 220 nm using a mixture of acetonitrile/ H_2O (80/20–65/35 % (0–15 min) and 65/35–25/75 % (15–30 min)) as the mobile phase, with a constant flow of 0.60 mL/min. Imidacloprid and dimethoate were also measured by HPLC/UV but using a reverse-phase column (Sunfire™ Waters C18 150–3 mm, 5 µm). The mobile phase was acetonitrile (15 %) and H_2O (85 %) in a concentration gradient to 80 % of acetonitrile and 20 % of H_2O in 18 min (flow rate 0.5 mL/min), and a wavelength of 220 nm was used. Cyclohexanone was quantified by gas chromatography with a flame ionization detector (GC 3900 Varian) using a 30 m long×0.25 mm i.d. capillary column (CP-Wax 52 CB) following the method described by Diaz et al. (2011).

Data handling

The results reported were the average values from duplicate runs, the standard errors always being lower than 10 %.

4.3. RESULTS AND DISCUSSION

Biodegradability of pesticides

Figure 4.1 shows the time course of MCPA, imidacloprid, dimethoate and cyclohexanone in the biodegradability tests. The maximum initial concentration of 500 mg/L used in these studies is consistent with maximum concentration of pesticides found in real industrial wastewaters (Buenronstro- Zagal et al., 2000; Chin et al., 2005). No significant losses by non-biological processes were observed along the experimentation. MCPA was partially removed (around 50 %) after 21 d regardless of the starting concentration used (Figure 4.1.a). In a previous work, González et al. (2006) studied the biodegradability of MCPA under aerobic conditions in membrane and fluidized biofilm biological reactors, the latter showing a faster acclimation of the

granular biomass. These aerobic systems led to an efficient removal of MCPA, up to 95 %. However, some other acidic herbicides like mecoprop and dichlorprop have been shown to be refractory to the anaerobic biological treatment (Zipper et al., 1999). MCPA was slowly removed by the anaerobic biomass, reaching an initial removal rate of 158.1 mg/gVS·d, which could be potentially accelerated in pure culture systems seeded with specialist degrading bacteria (Evangelista et al., 2010).

Imidacloprid remained unaltered during the biodegradability test (Figure 4.1.b), which suggests that neonicotinoid insecticides could appear in the resulting effluents from an anaerobic treatment. Some microorganisms isolated from soil have been shown to be efficient for a high-extend transformation of imidacloprid, although its mineralization is still a challenge (Pandey et al., 2009). The active species of the organophosphate insecticide was better removed, reaching a total depletion of dimethoate for concentrations lower than 100 mg/L (Figure 4.1.c). The increase of dimethoate concentration led to a clear reduction of the removal efficiency, suggesting the occurrence of a toxic effect over the anaerobic granular sludge. The biodegradability of cyclohexanone, as the major solvent contained in Danadim®, was also studied. As can be seen in Figure 4.1.d, this compound could be biodegraded within the entire concentration range studied in less than 15 h, reaching an initial removal rate up to 149.5 mg/gVS·d. There is scarce information about biodegradability of alicyclic compounds in the absence of molecular oxygen. Although the complete removal of cyclohexanone has been achieved by isolated facultative denitrifying bacteria under anaerobic conditions (Dangel et al., 1988), the anaerobic degradation of cyclohexanone has been mostly reported in presence of aromatic compounds. In some cases, the addition of cyclohexanone to the dimethoate-bearing insecticide increases its toxicity, concluding that solvents play a crucial role in dimethoate toxicity (Eddleston et al., 2012). This fact can make necessary the enhancement of the biodegradability of this highly toxic insecticide by a solar photocatalytic system, whose resulting easily

biodegradable intermediates would be subsequently mineralized in a bioreactor (Zapata et al., 2009). The analysis of the inhibition over the acetoclastic methanogenesis showed IC_{50} (half maximal inhibitory concentration) values of 474 and 367 mg/L for imidacloprid and dimethoate, respectively. IC_{50} values were not possible to obtain for MCPA and cyclohexanone since inhibition was not observed in the concentrations range tested.

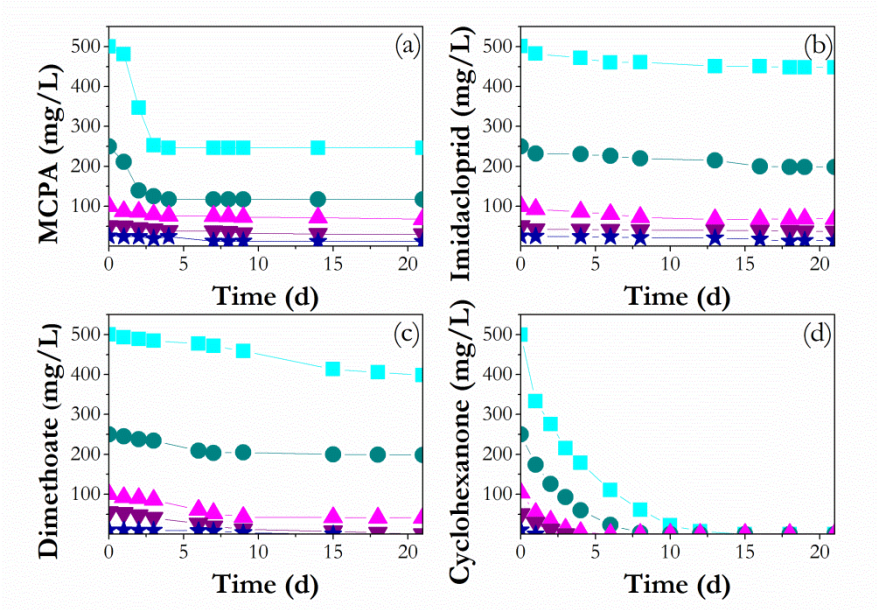

Figure. 4.1. Time course of pesticides active ingredients and cyclohexanone during the biodegradability batch tests for different starting concentrations: 10 (squares), 50 (circles), 100 (triangles), 250 (diamonds) and 500 mg/L (stars) of MCPA (a), imidacloprid (b), dimethoate (c) and cyclohexanone (d).

Since the chemical structure of intermediates generated upon the anaerobic treatment of commercial pesticides is normally unknown, TOC and COD were used to follow the mineralization process. The influence of active ingredient concentration on the mineralization of pesticides was analyzed (Figure 4.2). Although pesticides were not successfully removed during the biodegradability test, the concentration of TOC decreased in all cases (Figure 4.2.a), which

means that part of the organics of the pesticide formulations was completely removed. TOC removal efficiencies increased at increasing concentrations of the active species below 250 mg/L for imidacloprid and MCPA, and 100 mg/L for dimethoate. Higher concentrations led to a significant decrease of the mineralization extension, which suggests that pesticides could hinder the removal of the rest of the biodegradable organic matter present in the commercial pesticides. As can be seen in Figure 4.2.b, the presence of cyclohexanone in Danadim® (dimethoate) stimulated the anaerobic biomass activity, reaching the highest COD removal rates, which indicates the presence of easily biodegradable carbon source. However, the presence of dimethoate reduced the activity of the biomass, causing a decrease of the specific initial COD removal rate at concentrations higher than 250 mg/L of this organophosphate insecticide (Figure 4.2.b). MCPA and imidacloprid showed a similar trend in the specific initial COD removal rate. The COD removal observed during the treatment of MCPA could be partially due to the degradation of the active ingredient. In the case of the imidacloprid-bearing pesticide, the active ingredient remained unaltered, which suggests that some of the compounds present in the commercial pesticide were effectively degraded.

Continuous treatment in an EGSB reactor

Once the biodegradability of the toxicants had been studied in detail, a long-term treatment through an EGSB reactor was carried out to evaluate the applicability of this anaerobic technology to remove the pesticides tested. The EGSB reactor was operated for 90 d, a long start-up period being necessary for the adaption of the biomass (Figure 4.3). The addition of pesticides into the feed seemed to cause a drop of the COD removal efficiency. However, the reactor stabilized after 30 d, increasing the methane production from 0.4 to 0.9 gCH_4–COD/gCOD, indicating a sustainable recovery of the methanogenic activity. Then, a COD removal efficiency of 85 % was maintained along the experimental time.

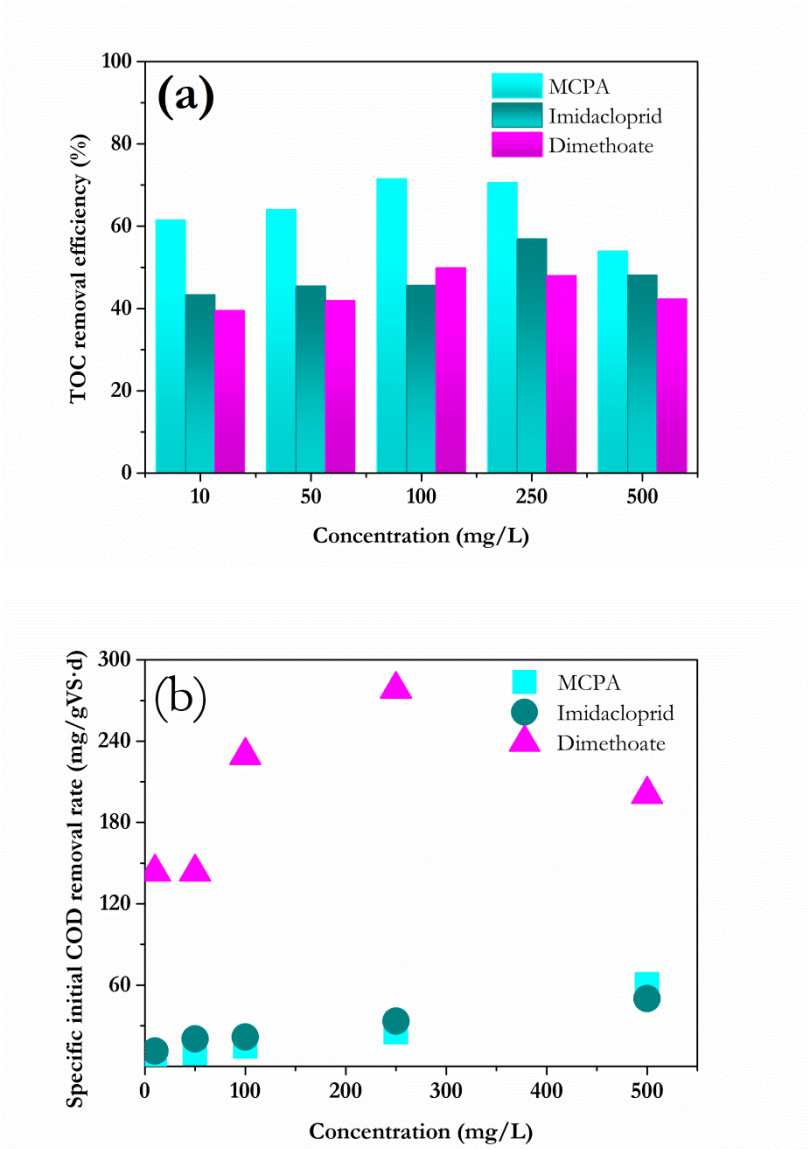

Figure 4.2. TOC removal efficiency (a) and specific initial COD removal rate (b) for different concentrations of the pesticides active ingredients after 21 d of anaerobic biodegradability test.

The high stability shown by the EGSB reactor implies that this technology is feasible for the treatment of pesticides bearing

wastewaters, adding valuable information regarding the EGSB applicability.

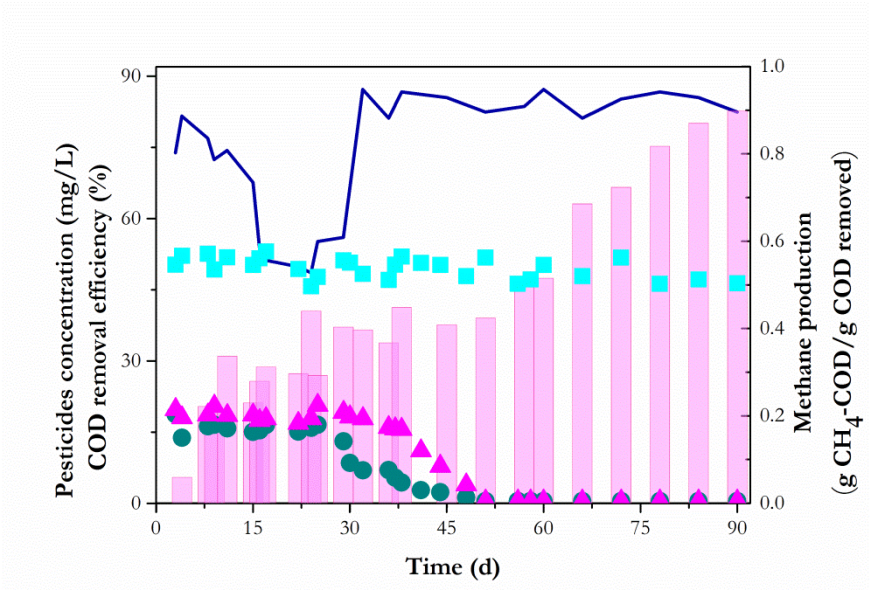

Figure 4.3. Time course of the COD removal efficiency (line), methane production (bars) and concentration of MCPA (squares), imidacloprid (circles) and dimethoate (triangles) detected in the effluents discharged from the EGSB reactor.

Cyclohexanone was not detected in the resulting effluents along the experimentation. The transformation of cyclohexanone into aliphatic acids and eventually to carbon dioxide and methane has been previously reported (Caldwell and Suflita, 2000). An acclimation period of around 50 d was needed to achieve the complete removal of imidacloprid and dimethoate. The improvement on the biodegradability of mixtures of pesticides could be caused by synergistic effects, as has been previously observed in acute toxicity bioassays (Fernández-Alba et al., 2002). The complete removal of these two pesticides gave rise to a significant increase in the COD removal and methane production efficiencies, indicating that the biodegradability increase improved the stability of the EGSB reactor. Although MCPA was partially removed in the biodegradability batch tests when the commercial selective herbicide was treated as sole

carbon source, a removal efficiency lower than 10 % was obtained when pesticides were fed together. The concentration of MCPA in the effluent remained constant along the continuous experiment. The low grade of substitution in the MCPA molecule could be a limiting factor for its removal in an enriched carbon medium. A previous work has reported a limiting biodegradation of monochlorinated species in anaerobic systems, being more adequate for the removal of poly-substituted molecules (Lopez et al., 2013). The N and P substitutions present in imidacloprid and dimethoate could benefit the efficient removal of these pesticides in the EGSB reactor. Therefore, the results from treating individual commercial pesticides are not really valid for predicting the kinetics of complex mixtures. In this sense, Feijoo et al. (1995) reported antagonistic effects for complex mixtures of several toxic and/or recalcitrant compounds on their biodegradability, so that specific studies are needed to analyze the applicability of anaerobic reactors to treat mixtures of pesticides present in wastewater.

4.4. CONCLUSIONS

MCPA and cyclohexanone have been succesfully biodegraded in batch experiments, while imidacloprid and dimethoate were not removed within 21 d of the trial. IC_{50} values for the acetoclastic methanogenesis were estimated to be 474 for Couraze® (20 % w/v imidacloprid) and 367 mg/L for Danadim® (40 % w/v dimethoate), respectively. MCPA and cyclohexanone caused a negligible inhibition over methanogenesis. Cyclohexanone was removed efficiently and remained undetectable in EGSB effluents. A long adaption time was required to reach the complete removal of imidacloprid and dimethoate. Thus, the overall removal efficiency of pesticides was enhanced under the operating conditions employed in the EGSB reactor. Mixtures of pesticides can be efficiently biotreated by anaerobic granular sludge in an EGSB reactor, reaching a high COD removal and a stable methane yield after the adaption of biomass to the presence of pesticides.

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5

COMMERCIAL PESTICIDES WASTEWATER TREATMENT USING AN ANAEROBIC HIGH-RATE REACTOR

5. COMMERCIAL PESTICIDES WASTEWATER TREATMENT USING AN ANAEROBIC HIGH-RATE REACTOR

Abstract

The degradation of commercial pesticides MCPA, Couraze® (imidacloprid) and Danadim Progress® (dimethoate) in batch and continuous experiment using a high-rate anaerobic reactor has been investigated preliminary. Biodegradability test of each active compound were carried out individually, observing a MCPA removal of 20 % after 45 d. Imidacloprid described a two-stage degradation model through the reduction of the nitro group. Dimethoate (organophosphate insecticide) was degraded within the concentration range studied (10–500 mg/L) following two possible pathways, the attack of the alkoxy group or the demethylation of the methylamine moiety. The biodegradability of binary and tertiary mixtures of the target pesticides showed that MCPA biodegradation was impeded in presence of other compounds, imidacloprid degradation was even improved but an acclimation phase was observed and dimethoate elimination was strongly affected. Methanogenesis was irreversible inhibited in batch test by adding imidacloprid and dimethoate which resulted in a reduction of the acetoclastic activity of 64 and 92 %, respectively. However, hydrogenotrophic activity was only decreased by dimethoate which caused an irreversible inhibition over the hydrogen-consuming archaea of 68 %. Continuous treatment was carried out in an expanded granular sludge bed reactor. COD removal efficiency of 65 %, methane production of 0.21 gCH_4-COD/gCOD consumed and removal efficiencies of 85 % of the selected pesticides were achieved when the active ingredients were added at concentrations of 87, 29 and 38 mg/L of MCPA, imidacloprid and dimethoate, respectively. The biomass identification highlights the

dominance of hydrogenotrophic archaea during the treatment in the EGSB reactor. SEM studies revealed no significant morphological changes in the sludge granules.

5.1. INTRODUCTION

Pesticides are widely used for control pests, including insects, rodents, fungi and unwanted plants (weeds). Agrochemical products contain active ingredient and a variety of solvents, synergists, surfactants, and other ingredients (inert ingredients) to improve the stability, delivery and effectiveness of the pesticidal ingredient (Surgan et al., 2010). These compounds must be used safely and disposed of properly (http://www.who.int/topics/pesticides/en/) because some of them are potentially toxic to other organisms, including humans. Several pesticides have been declared as priority pollutants in the EU legislation (Directive 2008/105/EC). The European Union Groundwater Directive (98/83/EC) establishes maximum allowable concentration of all individual active compounds of 0.1 μg/L in drinking water, being the sum of all pesticides concentration less than 0.5 μg/L. Highly polluted effuents with pesticides are mainly produce in washing center of pesticides containers and application equipment, agricultural industries and manufacturing pesticides plant, reaching pesticides concentrations of 500 mg/L (Chiron et al., 2000). The industrial wastewater generated in the washing of commercial pesticide containers is characterized by a heterogeneous composition in terms of variable concentration of dissolved organic carbon (DOC) (200–500 mg/L), chemical oxygen demand (COD) (1662–1960 mgO_2/L), biological oxygen demand (BOD_5) (1350–1600 mgO_2/L) ionic concentration and pesticide content (Zapata et al., 2010; Vilar et al., 2012).

2-methyl-4-chlorophenoxyacetic acid (MCPA), imidacloprid and dimethoate are pesticides widely employed in intensive agriculture. The use of MCPA, imidacloprid and dimethoate are regulated by Directives 2007/27/EC, 2006/26/EC and 2002/71/EC, respectively.

MCPA is one of the most representative phenoxy herbicides which are worldwide used for crops protection due to the disruption of the basic metabolic processes in plant cells and tissues (Grabinska-Sota, 2003). MCPA can be removed by chemical oxidation process as sole technology or combining it with biological degradation and adsorption systems (Reynolds et al., 1989; Gimeno et al., 2003; Brillas et al., 2004; García-Segura et al., 2011; Sanchis et al., 2013; Rívas et al., 2015; Solís et al., 2015). Its aerobic biodegradation have been effective by *Pseudomonas* (Kilpi et al., 1980), *Comamonas* (Muller et al., 1999), *Alcaligenes* (Pieper et al., 1988), *Sphingomonas* sp. (Nielsen et al., 2015) and even activated sludge (Sanchis et al., 2013). Also, it has been assessed different strategies for its removal as bioaugmentation in sand with formulated *Sphingobium* sp. T51 (Onneby et al., 2014). There are some publications that refer to the recalcitrant character of this phenoxy acetic acid under anaerobic conditions (Harrison et al., 1998; Battersby and Wilson, 1988). However, there is limited information about the MCPA degradation by anaerobes. Buisson et al. (1968) observed that MCPA was completely removed in anaerobic biodegradability batch test during 32 d at an initial concentration lower than 125 μg/L. Nevertheless, several articles have reported that other phenoxyacetic acids such as 2,4-dichlorophenoyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) can be removed anaerobically (Chang et al., 1998b; Zipper et al., 1999; Celis et al., 2008). Under anaerobic conditions 2,4-D is degraded by conversion to 2,4-dichlorophenol before dehalogenation occurred, whereas 2,4,5-T was first dehalogenated to form 2,5- or 2,4-dichlorophenoxyacetic acid which were converted to the corresponding dichlorophenols and subsequently dehalogenated further to monochlorophenols and to phenol (Gibson and Suflita, 1996).

Imidacloprid, (1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine), is a neonicotinoid insecticide that disrupts the insect nervous system (Cresswell et al., 2011). The oxidative degradation of imidacloprid has been carried out by electrochemical advanced oxidation processes (Turabik et al., 2014), ultrasound techniques alone

or combined with advanced Fenton process and/or UV based systems (Patil et al., 2014). However, this insecticide can be also metabolized by facultative anaerobic microorganisms like *Bacillus thuringiensis* and *Klebsiella pneumoniae*, reaching a removal efficiency of about 78 % of imidacloprid in 10 d (Phugare et al., 2013; Ferreira et al., 2015). In both publications imidacloprid was degraded to 6-chloronicotinic acid via formation of nitrosoguanidine and imidacloprid guanidine metabolites. *Bacillus weihenstephanensis* and *Paenibacillus polymyxa* have been also reported to be capable of remove up to 46 and 78 % of imidacloprid in minimun salts and tryptic soya medium, respectively (Arif et al., 2012; Shetti et al., 2014)

Dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate) is an organophosphorus insecticide. It is used on crops for the control of a broad range of insects (Li et al., 2010). Dimethoate can be mineralized by advanced oxidation processes (Oller et al., 2005), ultrasonic irradiation (Chen et al., 2007) and combining photo-fenton and biological oxidation (Ballesteros Martin et al., 2008, 2009). Several bacteria are capable of degrading dimethoate completely in 8 d such as *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Proteus mirabilis*, *Bacillus pumilus* and *Bacillus megaterium* (Deshpande, 2001; DebMandal et al., 2011).

High-rate anaerobic technologies have been improved significantly in the last few decades, which are now capable to treat hardly biodegradable wastewaters containing toxic compounds or with a complex composition (van Lier et al., 2015). One of the most demanded anaerobic systems for industrial wastewater treatment is the Expanded Granular Sludge Bed (EGSB) reactor (van Lier, 2008). EGSB reactors offer a smaller footprint, high mixing due to the high upward-flow velocities and consequently improved mass transfer, biomass activity and better transport of substrate into sludge aggregates. The reactor features high organic and hydraulic loadings, especially for acidified wastewater under psychrophilic conditions,

even at temperatures as low as 10 ºC. EGSB reactors are also suited for the treatment of medium strength wastewaters (COD < 1 g/L) (Mao et al., 2015). EGSB-systems possess a significant better potential for removing toxic biodegradable compounds like lauric and capric acids, higher fatty acids, formaldehyde and/or complex compounds present in industrial effluents (Lettinga, 2010). These characteristics make this technology feasible and sustainable for the treatment of toxic polluted wastewaters.

The aim of this work is to assess the anaerobic treatment of synthetic wastewater bearing commercial pesticides (MCPA selective herbicide, Couraze® and Danadim Progress®) by an EGSB reactor to optimize the operation conditions. The acetoclastic and hydrogenotrophic inhibition and/or toxicity caused by the target compounds have been also evaluated. In addition, a possible degradation pathway of the pesticides studied under anaerobic conditions have been proposed.

5.2. MATERIALS AND METHODS

Wastewater composition

Synthetic wastewater was prepared by adding the following components (mg/L): peptone (17.4), yeast extract (52.2), milk powder (116.2), sunflower oil (29), sodium acetate (79.4), starch (122) and urea (91.7) giving a chemical oxygen demand (COD) around 1.75 g/L. The fed wastewater was supplemented with 20 mL/L of the following macronutrient solution (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328), $MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and yeast extract (4). This medium was supplemented with 1 mL/L of the subsequent micronutrients solution (µg/L): $FeCl_2 \cdot H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO \cdot 5H_2O$ (162), EDTA (1,000), resazurin (0.2) and sulphuric acid 36 % (1 µL/L). $NaHCO_3$ was added (1 g/gCOD) as a buffer and alkalinity source. Pesticides were spiked in the feed as a fraction of total organic carbon (TOC) of 20 %. Once the steady state

was reached, pesticides concentration was increased gradually to 30 and 40 % (TOC fraction) at days 90 and 285, respectively.

Chemicals

The composition of the commercial pesticides studied is shown in Table 5.1.

Table 5.1. Commercial pesticides composition.

Commercial name Supplier	Active ingredient (% w/v)	Inert ingredients (% w/v)
Selective herbicide (Fertiberia, Spain)	MCPA (60)	Unknown
Couraze® Neonicotinoid insecticide (Cheminova Agro S.A., Spain)	Imidacloprid (20)	Dimethylsulphoxide (60) Propylene carbonate (20)
Danadim Progress® Organophosphate insecticide (Cheminova Agro S.A., Spain)	Dimethoate (40)	Ciclohexanone (43) Xilene (13) Maleic anhydric (1)

Biomass source and characterization

Anaerobic granular biomass was collected from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating beet sugar wastewater (Valladolid, Spain). The granules had an average diameter of 0.5 mm and a specific methanogenic activity (SMA) of 0.269 (0.006) gCH_4-COD/gVS·d.

Anaerobic biodegradability, methanogenesis inhibition and toxicity assays

Anaerobic batch tests were performed inoculating 1.5 g volatile solids (VS)/L of non-adapted granular sludge. The experiments were carried out at 30±1 °C in duplicate, using the Automatic Methane Potential Test System (AMPTS, Bioprocess Control, Sweden) as reported by Garcia-Mancha et al. (2012). Cyclohexanone, present in Danadim Progress®, was chosen to evaluate the effect of an inert ingredient over the anaerobic biomass.

Anaerobic biodegradability tests of commercial pesticides were performed individually and in mixtures for 45 and 28 d, respectively. As sole carbon source each commercial pesticide was added at different concentration ranging from 10 to 500 mg/L of active ingredient. Biodegradability of binary and tertiary mixtures of pesticides was carried out at a relative TOC concentration of 20 % of the feed (58 mg/L MCPA, 20 mg/L imidacloprid and 25 mg/L dimethoate).

The inhibition and toxic effect of pesticides over acetoclastic and hydrogenotrophic methanogenesis was studied according to the following three steps protocol: first, the anaerobic acetoclastic and hydrogenotrophic biomass present in the sludge was activated adding sodium acetate (4 g/L) or sodium formiate (2 g/L), respectively. Then, sodium acetate/formiate and pesticides (at different active ingredient concentration from 10–500 mg/L) were added to evaluate the inhibition potential. Finally, biomass was collected and sodium acetate or formiate was added as sole carbon source to evaluate the reversibility of the inhibition effect.

Abiotic test

The contribution of adsorption was evaluated in biomass samples after extraction with Soxhelt following the US-EPA 8041 method. Tests of volatilization were performed under identical operating conditions to those in the biodegradation experiments but in the absence of biomass. The results reported are the average values from duplicate runs, the standard errors being always lower than 10 %.

Experimental set-up for continuous runs

Experiments in continuous mode were carried out in a 5.2 L EGSB reactor with an internal diameter to height ratio of 1:7.2. The reactor was equipped with a gas–liquid–solid separator installed 15 cm below the exit. Details of the apparatus are described elsewhere (Monsalvo et al., 2014). The reactor was operated at an upward flow rate of 2.5 m/h, mesophilic condition (35 °C) and a hydraulic retention time of 1 d. The EGSB reactor was inoculated with 100 gVS of granular sludge

previously activated adding the synthetic feed without pesticides until steady stage was reached. Macro- and micronutrients, as well as $NaHCO_3$ (buffer and alkalinity source) were supplemented as indicated above. An organic loading rate (OLR) of 1.75 gCOD/L·d were maintained constant during long-term continuous experiment (408 d). The reactor was operated at different active ingredients loading rates (AILR) as gAI/L·d by increasing their relative TOC fraction in the feed from 20 to 40 %. Table 5.2 summarizes the different operating conditions.

Table 5.2. Operating conditions in the EGSB reactor for the continuous experiment.

Stage	t (d)	TOC (%)	AI	AILR
I	0	20	MCPA	0.058
			Imidacloprid	0.020
			Dimethoate	0.025
			Cyclohexanone	0.027
II	90	30	MCPA	0.087
			Imidacloprid	0.029
			Dimethoate	0.038
			Cyclohexanone	0.041
III	285	40	MCPA	0.100
			Imidacloprid	0.039
			Dimethoate	0.050
			Cyclohexanone	0.054

Analysis of biomass profile by denaturing gradient gel electrophoresis (DGGE)

The phylogenetic of the biomass profile has been characterized along the anaerobic processes following a standardized denaturing gradient gel electrophoresis (DGGE) protocol where the bacterial and archaeal communities have been targeted independently. Granular sludge was extracted from the EGSB reactor at the beginning and at the end of each operation stage (90 and 285 d). The sludge was resuspended in PBS 1X (pH 7.0), and cells were disrupted using a BIO101-Savant FP120 cell disrupter (Q-BIOgene, Carlsbad, CA, USA) (six times for

40 s, each at 5.5 cycles/s). DNA extraction, amplification and purification protocols as well as the DGGE procedure, were performed as previously described by Garcia-Mancha et al. (2012). The sequences were compared with those listed in the GenBank nucleotide sequence databases using Chromas 2.0 software. The BLAST search option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database. Determination of the taxonomical hierarchy was performed using the Classifier tool from the Ribosomal Database Project (RDP) web page (http://rdp.cme.msu.edu/index.jsp) for the entire DNA sequences.

Scanning electron microscope (SEM) images

The morphology of anaerobic granules was characterized by SEM using a digital Phillips XL 30 apparatus according to a method previously reported (Alphenaar et al., 1994).

Analytical methods

Analyses of total and soluble chemical oxygen demand (TCOD, SCOD), total and volatile suspended solids (TSS, VSS) were performed according to the APHA Standard Methods (APHA, 1992). TOC was measured by a Shimadzu TOC-VCPH/CPN analyzer.

The quantification of the pesticides active ingredients (MCPA, imidacloprid and dimethoate) species was performed by high-performance liquid chromatography (HPLC) (Varian Prostar 325, Santa Clara, CA, USA) with a UV detector using a reverse-phase column (Agilent Technologies, Microsorb MV 100-5 C18 250×4.6 mm). The mobile phase was acetonitrile (15 %) and H_2O (85 %) in a concentration gradient to 80 % of acetonitrile and 20 % of H_2O in 27 min (flow rate 0.5 mL/min). A wavelength for MCPA and dimethoate of 220 nm and 270 nm for imidacloprid was used. Cyclohexanone and cyclohexanol were quantified by gas chromatography with a flame ionization detector (GC 3900 Varian, Santa Clara, CA, USA) using a 30 m long×0.25 mm i.d. capillary column (CP-Wax 52 CB, Varian) and nitrogen as carrier gas. The following temperature program was

used: starting temperature 70 ºC, heating rate 15 ºC/min and final temperature 240 ºC. The temperature of the detector was always 300 ºC.

Intermediates were analyzed by liquid chromatograph-electrospray mass spectrometry (LC – ESI - MS) (Agilent 6120 single quadrupole MS detector coupled to a Agilent 1100 series HPLC, Agilent Technologies, Santa Clara, CA,USA) operating in negative mode under the following conditions: Gemini 150×4.6mm, 5µm (Phenomenex) and a gradient of acetonitrile and formic acid/water [(0 min) 15 %/85 % ACN:0.1 % (v/v) formic acid/H_2O; (3 min) 30 %/70 % ACN:0.1 % (v/v) formic acid/H_2O; (6 min) 40 %/60 % ACN:0.1 % (v/v) formic acid/H_2O; (12 min) 50 %/50 % ACN:0.1 % (v/v) formic acid/H_2O; (15 min) 80 %/20 % ACN:0.1 % (v/v) formic acid/H_2O; (20–50 min) 15 %/85 % ACN:0.1 % (v/v) formic acid/H_2O].

5.3. RESULTS AND DISCUSSION

Biodegradability of commercial pesticides

Figures 5.1 and 5.2 show the time course of active and inert components concentration of commercial pesticides: MCPA (Figure 5.1.a), Couraze® (Figure 5.1.b) and Danadim Progress® (Figure 5.2.a and 5.2.b) during the biodegradability test. The specific initial MCPA removal rate increased from 0.094 to 3.76 mg/gVS·d at increasing concentrations up to 100 mg/L. However, MCPA removal rate decreased at starting concentrations higher than 250 mg/L. MCPA removal efficiency diminished from 30 to 18 % when MCPA initial concentration was increased from 10 to 100 mg/L. This fact suggests the occurrence of inhibition effect. No CH_4 was produced during the biodegradability test of MCPA, highlighting that the slightly decreased in MCPA concentration corresponded with its transformation instead of its mineralization.

Buisson et al. (1968) observed that MCPA was completely removed by anaerobes after 32 d treating initial concentrations up to 125 μg/L. In contrast to this, numerous publications have concluded that MCPA is a recalcitrant compound, like other phenoxy acetic pesticides towards the biodegradation under anaerobic conditions (Harrison et al., 1998; Battersby and Wilson, 1988).

As other chlorinated phenoxyacetic acids (2,4-D and 2,4,5-T), the degradation of MCPA under anaerobic conditions usually occurs by dehalogenation. The dehalogenation time varies between 4 weeks and 4 months, and it can be potencially reduced by the addition of other carbon source (Gibson and Suflita, 1986; 1990; Boyle et al., 1999).

Imidacloprid biodegradation started with a fast initial degradation phase followed by a very slow degradation and a final reactivation phase, describing a two-stage, or biphasic model (Figure 5.1.b). Broznić et al (2011) explained this degradation process in soils by the existence of different compartments where degradation proceeds at different rates. The fast degradation in the first compartment occurs when the pesticide is in the soil-water phase and readily available for microorganisms. In the second compartment the pesticide is sorbed to soil particles. In the present work the granule can be divided in different compartment located in the surface of the granule and inside of it with different metabolic activities. However, other mechanisms are also possible. For example, microorganism populations may increase or decrease over time (Sarmah and Close, 2009). Another explanation for this phenomenon could be that a decline in microbial viability, due to that the microbial population could be stressed during the experimental period, may have contributed to the decrease in degradation rate (Strek, 1998).

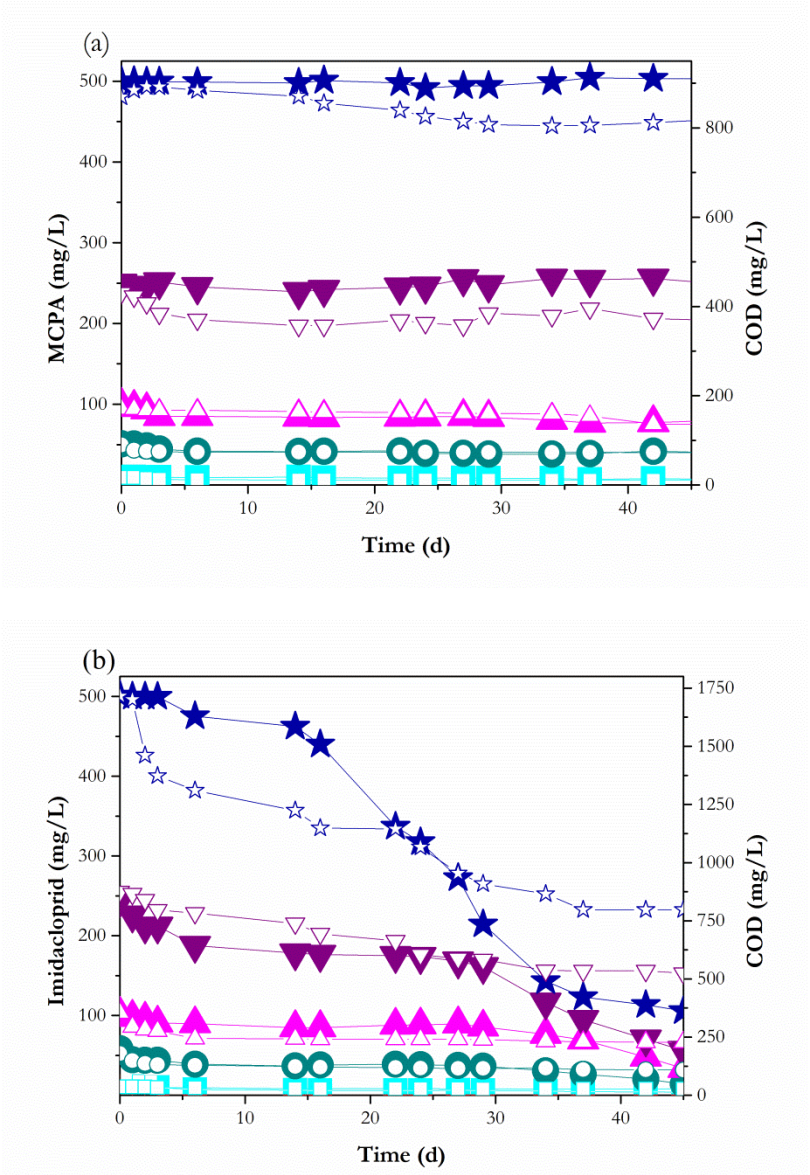

Figure 5.1. Time-evolution of MCPA (a) and imidacloprid (b) (solid symbols) and COD removal efficiency (open symbols) at different initial concentrations of commercial pesticides.

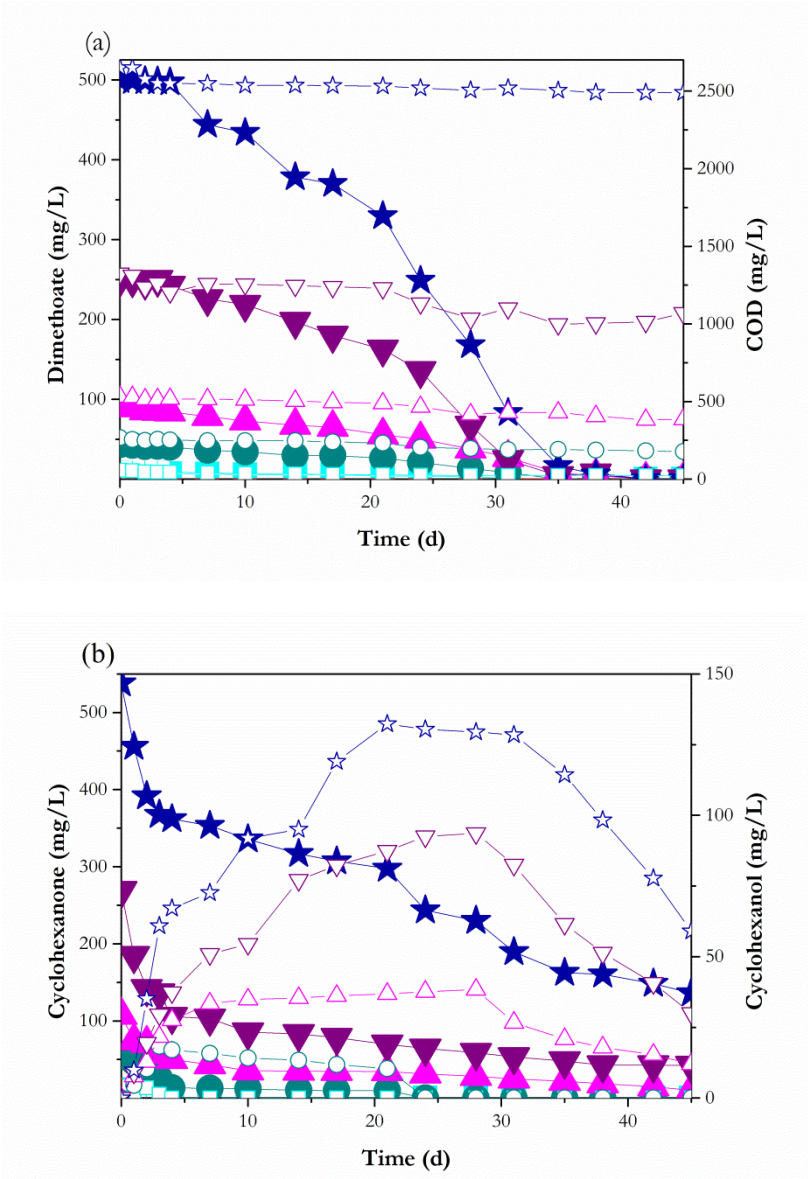

Figure 5.2. Time-evolution of (a) dimethoate (solid symbols) and COD removal efficiency (solid symbols) and (b) cyclohexanone (solid symbols) and cyclohexanol (open symbols) at different initial concentrations of commercial pesticides.

Due to that imidacloprid disappeared could occurs through different process as biodegradation, biosorption and diffusion into the granule

structure (Capri et al., 2001). The initial imidacloprid degradation rate increases at increasing initial concentration from 0.102 to 12.963 mg/gVS·d. Only when the starting concentration was 500 mg/L an acclimation phase of 3 d was observed. Imidacloprid and COD removal efficiencies increased slightly with increasing initial concentration, from 64 to 89 % and from 26 to 53 %, respectively. The increase in imidacloprid degradation with the concentration could be related with the activation of the genes responsible of it degradation which requires high concentrations to be induced (Helbling, 2015). The COD removal can be in part attributed to the inert biodegradable fraction (dimethylsulfoxide and propylene carbonate) in Couraze® formulation. Nevertheless, CH_4 was no produced. Gunsalus and Wolfe (1978) have suggested that the final stage in the methanogenic pathway (conversion of CH_3-S-coenzyme M to CH_4) is the step susceptible to pesticide action. The COD decrease could be due to the hydrolysis and acidification stages in anaerobic digestion in which insignificant or no CH_4 production means good fermentation process (Ince, 1998; Parawira et al., 2008)

The metabolites obtained during imidacloprid biodegradation were characterized by LC–ESI-MS studies. Imidacloprid was transformed into several intermediates which were identified as nitrosoguanidine (MW: 240), desnitro/guanidine metabolite (MW: 211) and urea metabolite (MW: 210) according to the degradation pathway proposed by Pandey et al. (2009). A subsequent opening of the imidazolidine ring of desnitro/guanidine metabolite or the loss of HCN from imidacloprid urea could formed an unknown intermediate (MW: 184) with two possible structures as proposed by Ding et al. (2011). The possible degradation pathway of imidacloprid is despited in the Figure 5.3.

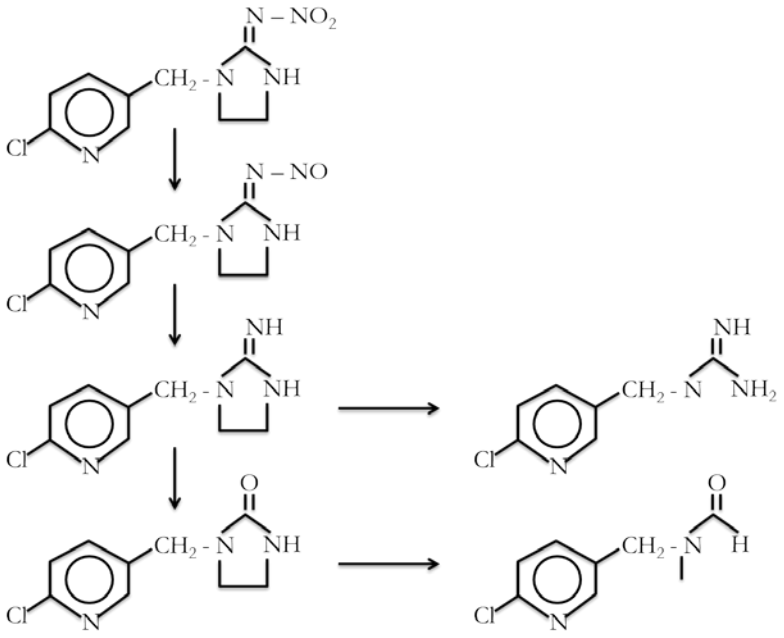

Figure 5.3. Imidacloprid degradation pathway under anaerobic conditions.

As can be seen in Figure 5.2.a dimethoate was removed within the concentration range studied. Dimethoate removal profile showed a slow initial removal rate followed by a fast removal stage. The removal rates increased at increasing dimethoate initial concentrations, reaching values of 5.84 and 13.22 mg/gVS·d for the slow and fast stage, respectively. In Danadim Progress® commercial product, cyclohexanone was identified as the major solvent and cyclohexanol was detected as the main degradation product resulted from its reduction reaction. Removal efficiencies of cyclohexanone and cyclohexanol (Figure 5.2.b) decreased at increasing of starting dimethoate concentration, which suggests the occurrence of inhibitory phenomena. Cyclohexanone and cyclohexanol were completely removed at dimethoate initial concentrations lower than 50 mg/L, while higher concentrations did not lead to their complete transformation. No COD removal and therefore no CH_4 production were observed along the trials.

According to the metabolites identified by LC-MS (MW: 229, 215, 185, 142 and 105) two degradation pathways can be proposed (Figure 5.4). Dimethoate cen be attacked hydrolytically at the alkoxy group obtaining O-methyl-O-hydrogen-S-(N-methylcarbamoylmethyl) phosphorodithioate (MW: 215), the des-methyl derivative of dimethoate. The alkoxy group is one of the sites proposed by Dauterman et al. (1959) for the initial attack of dimethoate. This intermediate was also identified by Dauterman et al. (1960) and Hacskaylo and Bull (1963). The next step according with the LC-MS analysis can be a second hydrolytic attack at the other alkoxy group and the oxidation of P=S bond. Subsequently the breaking of the bond P-S took place obtaining N-(methyl) mercaptoacetamide (MW: 105) and phosphoric acid which also was detected by Yao et al. (2011) and Hacskaylo and Bull (1963). Alternativelly, the second degradation pathway proceeds by a demethylation of the methylamide moiety of dimethoate to form de-N-methyl dimethoate (O,O-Dimethyl-S-carbamoylmethyl phosphorodithioate). Lucier and Menzen (1968) detected the resulting metabolite at trace amounts as a product of dimethoate degradation. Then, the conversion of P=S to P=O and the scission of the bond S-C took place, obtaining the metabolite MW: 142 also identified by Hu et al. (2013).

The degradation of cyclohexanone as sole carbon source was also studied (Figure 5.5). Cyclohexanone was completely removed up to 50 mg/L (Figure 5.5.a), reaching a maximum specific initial removal rate of 33.27 mg/gVS·d. Nevertheless, the concentration of COD remained constan (Figure 5.5.b) which suggests the cyclohexanone was transformed and intermediates accumulated in the medium. Accordingly CH_4 was not produced. These results are in accordance with those reported by Fang et al. 2006, who did not observed methane production after 25 d of specific methanogenic activity (SMA) test using cyclohexanone as substrate.

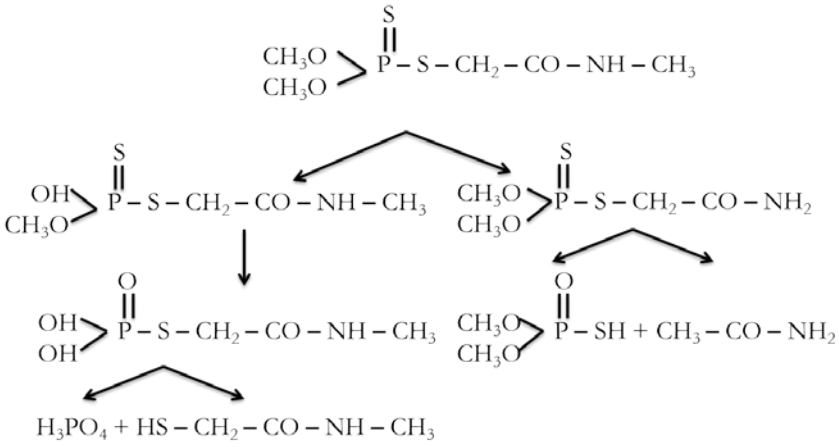

Figure 5.4. Dimethoate degradation pathway under anaerobic conditions.

Cyclohexanone can be metabolized by different microbial degradation pathways: (i) conversion to the corresponding lactones before cyclic cleavage, (ii) reduction to the corresponding alcohols and (iii) transformation to 2-hydrocyclohexane-1-one before hexane ring cleavage (Yoshizako et al., 1992). As aforementioned, cyclohexanol was detected during the cyclohexanone biodegradation experiments. Both cyclic compounds can be degraded under anaerobic conditions (Figure 5.6), forming aliphatic acids and eventually CO_2 and CH_4 (Grbic-Galic and Vogel, 1986; Evans, 1977).

Biodegradability of mixtures of commercial pesticides

Figure 5.7 shows the specific initial removal rate for the active ingredients contained in binary (Figure 5.7.a) and tertiary (Figure 5.7.b) mixtures. It was observed that MCPA was not degraded in presence of other pesticides. There are only a few studies about the behavior of these compounds in complex mixtures with other pesticides. Marriot et al. (2000) reported that the MCPA degradation rate decreased in binary and tertiary mixtures with other chlorophenoxy alkanoid acids herbicides (MCPA, 2,4-D and MCPP).

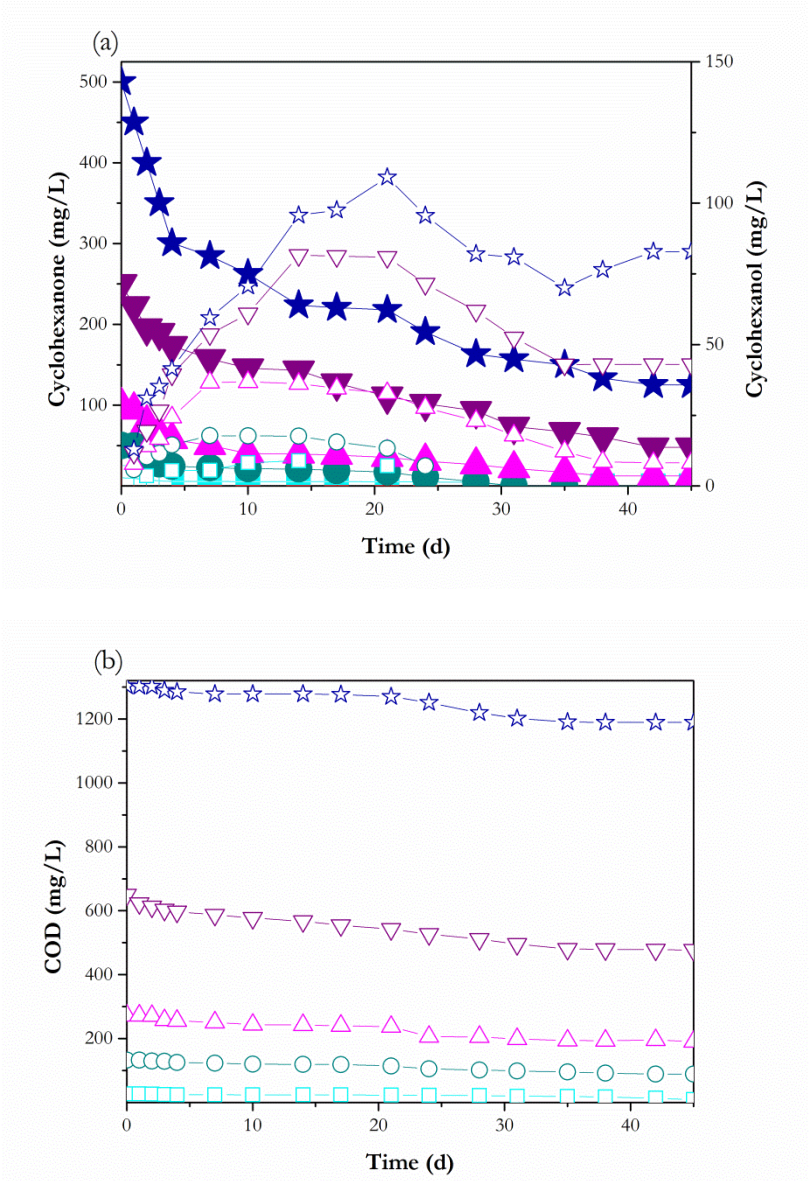

Figure 5.5. Time course of (a) cyclohexanone (solid symbols) and cyclohexanol (open symbols) and (b) COD removal efficiency during cyclohexanone biodegradability assay as sole carbon source.

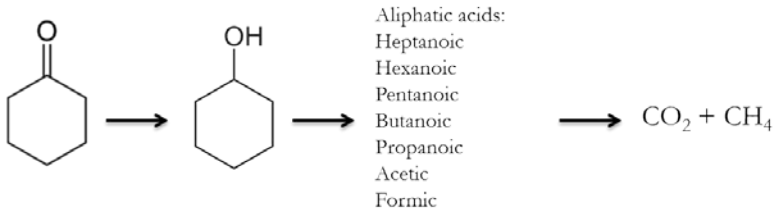

Figure 5.6. Cyclohexanone degradation pathway under anaerobic conditions.

Strachan et al. (2001) studied the toxicity of mixtures of herbicides in freshwater to evaluated interactions between them. The results showed that the toxicity of MCPA bearing solutions depends on chemical properties of the rest of compounds, whose biodegradability is also affected by the presence of MCPA.

Imidacloprid was effectively removed in presence of other compounds, and its specific initial rate as sole pesticide (0.093 mg/g VS·d) increased in in binary mixtures (1.4, 1.2 and 0.9 mg/gVS·d in presence of MCPA, Danadim Progress® and cyclohexanone, respectively) and even more in tertiary blends (1.9, 1.6 and 1.1 mg/gVS·d in presence of MCPA+Danadim Progress®, Danadim Progress®+cyclohexanone and MCPA+cyclohexanone, respectively). An initial acclimation period of around 8 d was observed in most of the cases, which was extended to 17 d when adding MCPA. The presence of a variety of organic pollutants can enhance the degradation of imidacloprid because of the availability of additional carbon sources (Abraham et al., 2014) which induces different enzymes like cytochrome P450, inhibition and detoxification enzymes (Dubois, 1969; Pape-Lindstrom and Lydy, 1997). There is a dearth of scientific information regarding biodegradation of imidacloprid in pesticides bearing mixtures, whose toxicity depends on the mixture composition. Imidacloprid toxicity increased when it is combined with nonylphenyl polyethoxylate, fipronil and atrazine (Key et al., 2007; Chen et al., 2010). However, when imidacloprid was added only with atrazine or only with fipronil no change in toxicity was observed (Key et al., 2007).

Dimethoate was slowly degraded in presence of other pesticides, achieving removal efficiencies between 20 and 46 % depending on the mixtures composition. Cyclohexanone was removesd between 79 and 89 %. Both, dimethoate and cyclohexanone initial removal rates decreased in presence of other substances, especially with imidacloprid and in tertiary mixtures. Xiaoqiang et al. (2007) reported that the presence of a pollutant can inhibit the degradation of a second compound. Thus, other pesticides may alter the degradation behavior of dimethoate through its effect on the dimethoate degrading microbes. For instance, Anderson and Zhu (2004) reported that atrazine induces cytochrome P450 monooxygenases which enhance the oxidative activation of dimethoate into more toxic O-analog metabolites resulting in elevated acetylcholinesterase (enzymes that catalyzes the hydrolysis of the neuro transmitting agent acetylcholine (Fukuto, 1990)) inhibition and increased toxicity. This fact can explain the decrease of dimethoate removal rate in presence of other pesticides. According to Helbling (2015), other probable reason is the occurrence of the carbon catabolite repression phenomenon, in which the expression of genes and enzymes, responsible of metabolize one carbon source (dimethoate), decrease when a preferred substrate (imidacloprid) is added. The preferred pesticide was imidacloprid followed by dimethoate and the last one was MCPA.

Methanogenesis inhibition

Inhibitory and toxic effects over the methanogenic biomass (acetoclastic and hydrogenotrophic) were investigated. Figures 5.8.a and 5.8.b and represent the acetoclastic inhibition observed with the pesticides at different concentration by showing the normalized activity (LCH_4/gVS·d), on the left, and the normalized total methane production (LCH_4) on the right. Figure 5.9.a and 5.9.b described the recovery level (reversible or irreversible inhibition) when the pesticides were taken off the medium and the sole carbon source was acetate.

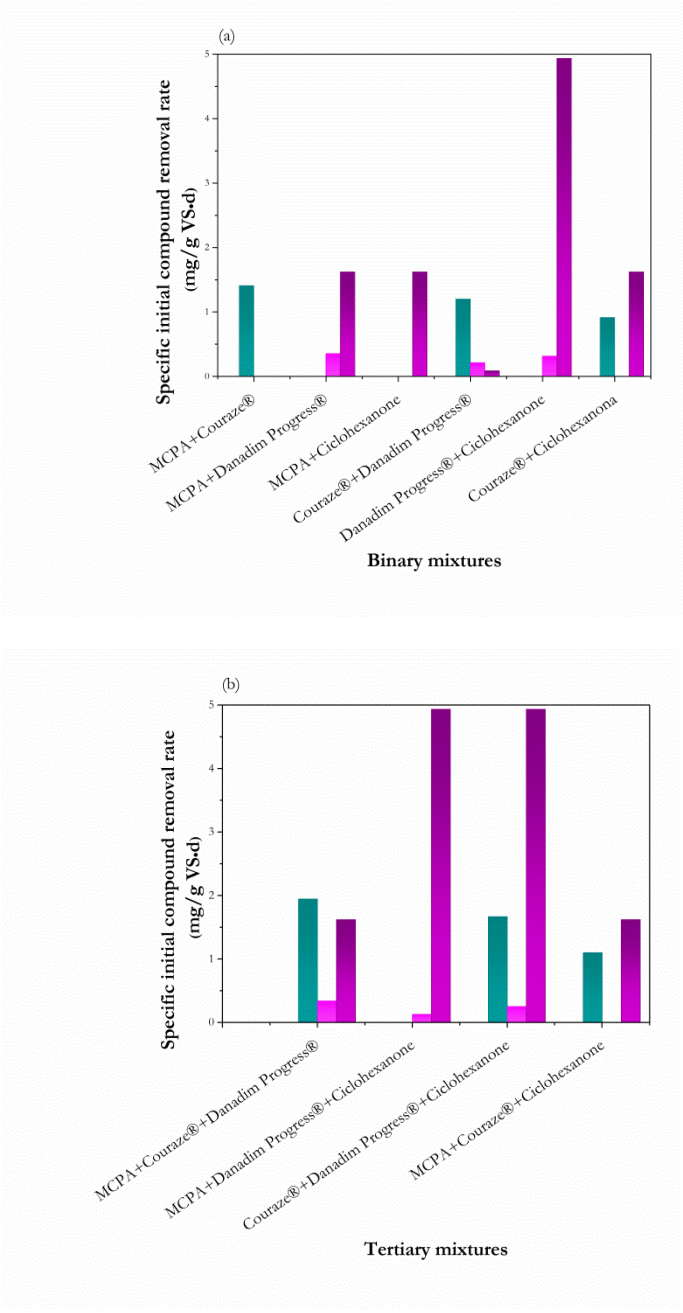

Figure 5.7. Specific initial removal rate of MCPA, imidacloprid (dark cian), dimethoate (magenta) and cyclohexanone (purple) treating different binary (a) and tertiary (b) mixtures of commercial pesticides.

Similarly, Figures 5.10 and 5.11 show the results over hydrogenotrophic biomass in presence of the target pesticides and the recovery grade adding formate as sole carbon source.

MCPA did not affect the acetoclastic archaea. However, MCPA and/or the corresponding inert ingredients improved the hydrogenotrophic activity. There is limited information about the inhibition of MCPA over methanogenic biomass, and only Battersby and Wilson (1989) found that MCPA was inhibitory in the initial phase of incubation with anaerobic digesting sludge, but this inhibition was overcome after 6 weeks.

Imidacloprid (Couraze®) concentrations higher than 250 mg/L led to a decrease of the acetoclastic activity (Figure 5.8.a), which was not recovered when these compounds were removed from the medium (Figure 5.9.a). Total methane production remained unaltered regardless the initial concentration of imidacloprid. Imidacloprid and/or the corresponding inert ingredients also improved the hydrogenotrophic activity. Although the biodegradation of imidacloprid by various bacteria species (*Bacillus* sp., *Brevibacterium* sp., *Pseudomonas putida* F1, *Bacillus subtilis* and *Rhizobium* sp.) was reported (Sabourmoghaddam et al., 2015), no evidence of its effects over acetoclastic and hydrogenotrophic methanogenesis has been published so far. Nevertheless, Tisler et al. (2009) studied the toxicity of imidacloprid and its commercial product (Confidor SL 200) over aquatic organisms, concluding that the toxicity of this commercial insecticide was higher than the toxicity of the pure imidacloprid.

The acetoclastic activity increased when low concentrations of dimethoate (< 100 mg/L) were fed. However higher concentrations caused a dramatic drop of the biodegradation activity. Similarly ocurrs in terms of total CH_4 production, but the inhibition was not as pronounced as in the acetoclastic activity. Dimethoate was also toxic for the acetoclastic archaea, and the inhibitory effect did not disappeared when dimethoate was removed. Higher concentration of

dimethoate caused a strong decrease on total CH_4 production. Only dimethoate caused a toxic effect over the hydrogenotrophic archaea when concentrations higher than 100 mg/L were treated. Chakraborty et al. (2002) studied the effect of the pesticide Tara-909 (contains dimethoate as active ingredient) over the methane production from sewage sludge, finding that, even at concentrations of 5 μg/ml, it appeared to be highly toxic, especially for biomethanation.

Cyclohexanone had no effects over the acetoclastic archaea, moreover it enhanced the hydrogenotrophic activity, showing a maximum at 250 mg/L.

The difference observed in the biomass activity and total CH_4 production may be associated to several hypotheses: i) microbial population could be stressed during the experimental period which may have contributed to the decrease in degradation rate (Strek, 1998), ii) the presence of the inhibitory/toxic compounds could have a kinetic impact, thus, it would take more time for the completion of microbial activity ending with full utilization of available organic substrate and therefore producing the same amount of methane produced in the absence of inhibitory/toxic compound (Cetecioglu et al., 2012) and iii) microorganism populations may increase or decrease over time (Sarmah and Close, 2009).

As in previous works (Lopez et al., 2013, Monsalvo et al., 2013) hydrogenotrophic biomass were more resistant than the acetoclastic ones. This behavior has been attributed to the differences in metabolic pathways (inhibition of complex and coenzymes related with methane production from acetate) and the lack of a protective envelope around the cell wall (Chidthaisong and Conrad, 2000; Gerardi, 2003; Smith and Ingram-Smith, 2007).

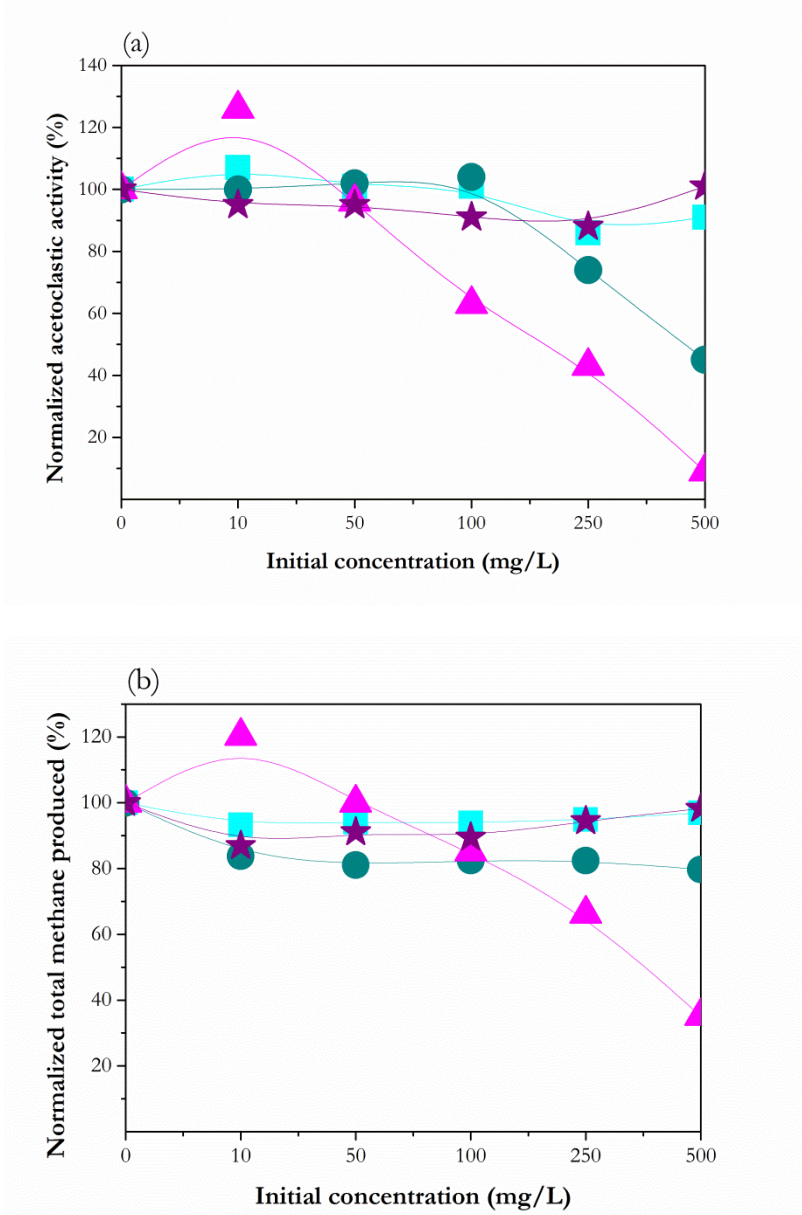

Figure 5.8. Normalized acetoclastic activity (a), and normalized total methane production (b) using different commercial pesticides (Selective herbicide MCPA (squares), Couraze® (circles) and Danadim Progress® (triangles) and cyclohexanone (stars)) during the inhibition.

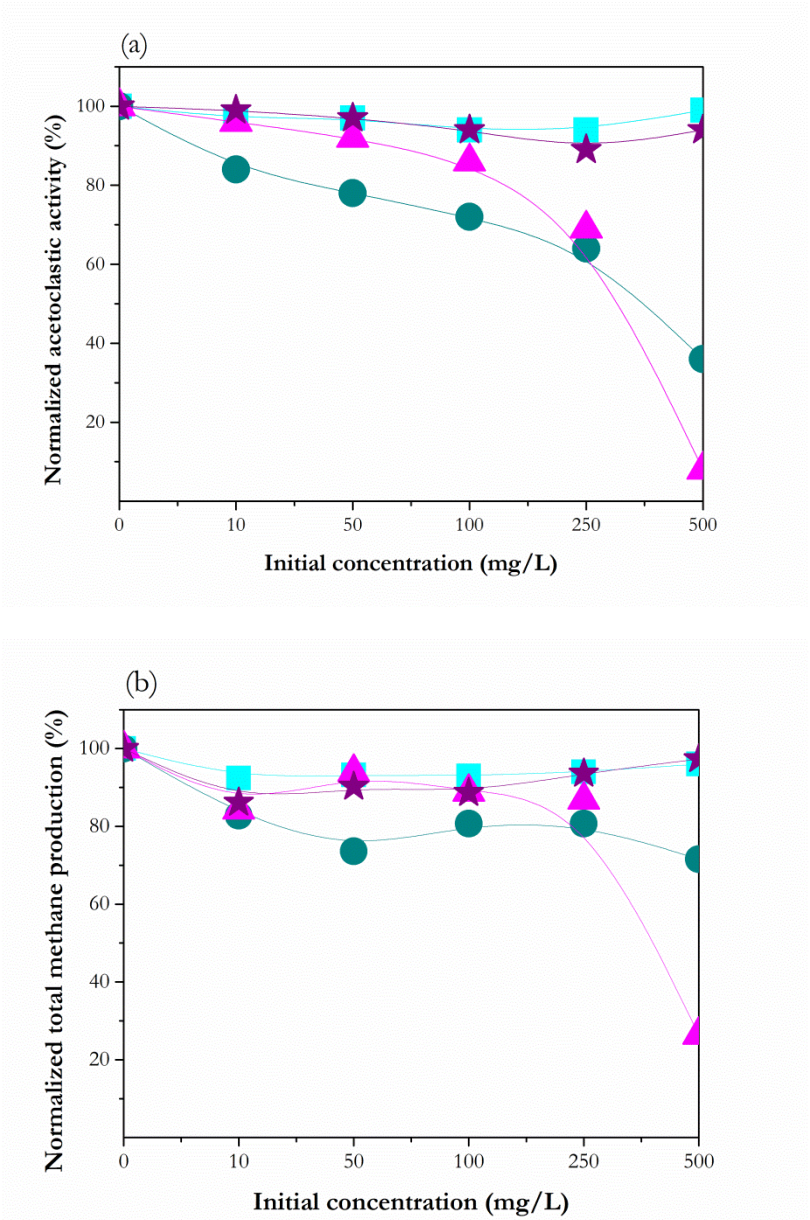

Figure 5.9. Normalized acetoclastic activity (a), and normalized total methane production (b) using different commercial pesticides (Selective herbicide MCPA (squares), Couraze® (circles) and Danadim Progress® (triangles) and cyclohexanone (stars)) during the recovery test.

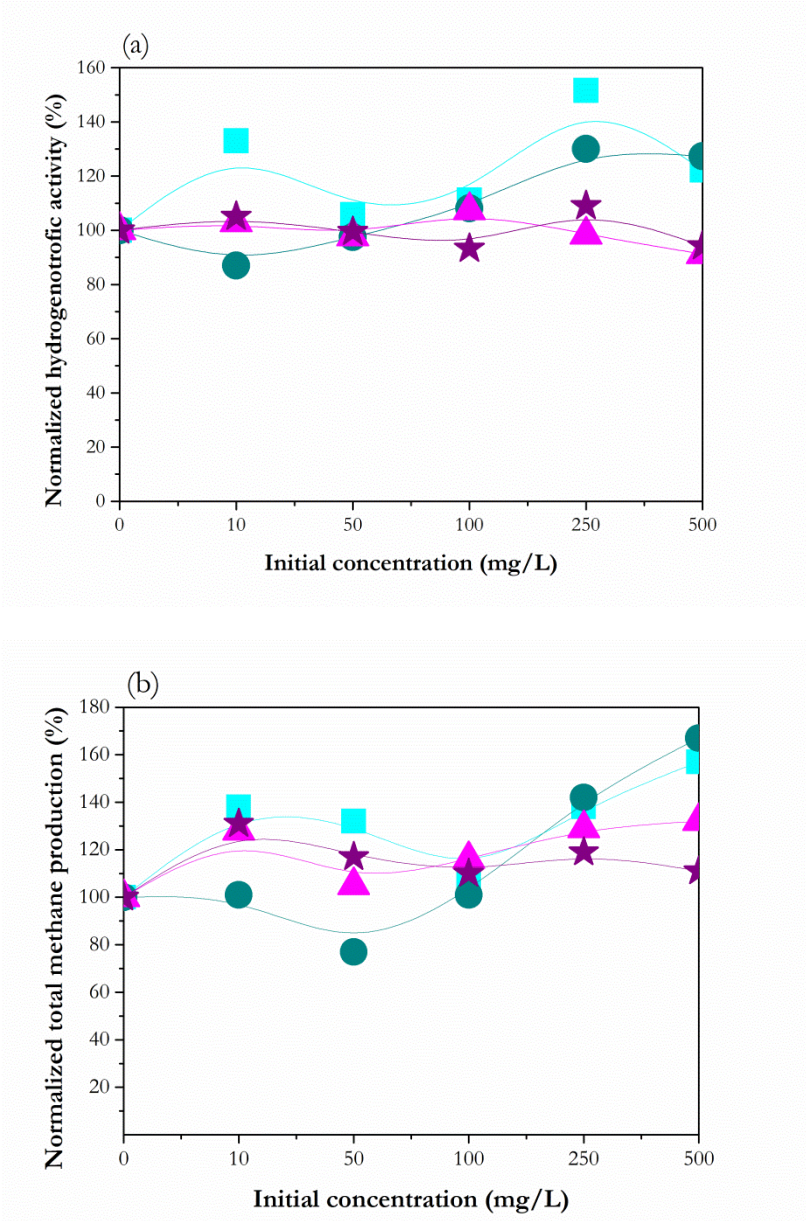

Figure 5.10. Normalized hidrogenotrophic activity (a), and normalized total methane production (b) using different commercial pesticides (Selective herbicide MCPA (squares), Couraze® (circles) and Danadim Progress® (triangles) and cyclohexanone (stars)) during the inhibition assays.

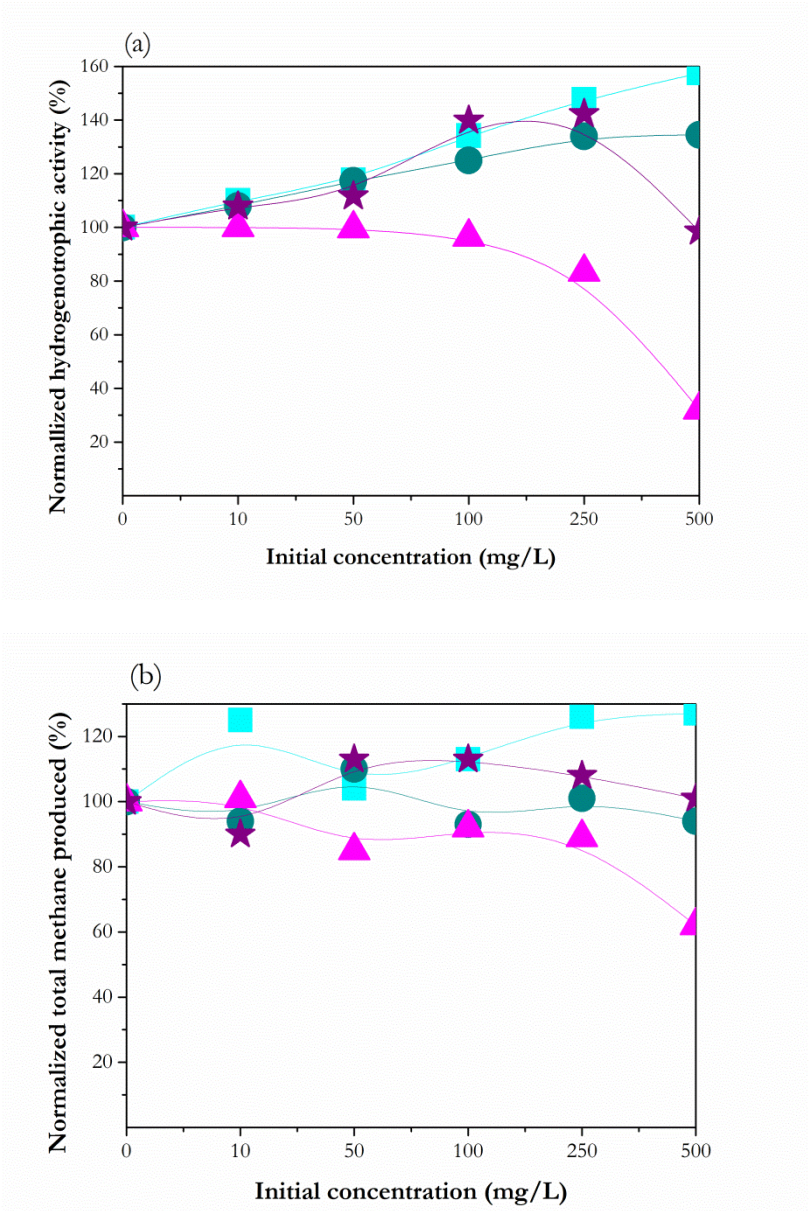

Figure 5.11. Normalized hidrogenotrophic activity (a), and normalized total methane production using different commercial pesticides (Selective herbicide MCPA (squares), Couraze® (circles) and Danadim Progress® (triangles) and cyclohexanone (stars)) during the recovery test.

Long-term continuos experiments

The performance of the EGSB reactor is shown in Figure 5.12. The system was operated according to the experimental plan described in Table 5.2, and COD removal efficiency, pesticides concentration in the discharge, as well as methane production were monitored.

Every change of pesticides concentration in the feed caused destabilization of the reactor behavior, especially COD removal efficiency and methane production. After that, a readaption period was observed before reaching steady state again. This readaption period was longer at increasing relative pesticides COD fraction. In the first stage 30 d were necessary until reaching a constant COD removal efficiency (86 %), in the second stage the re-adaption time took 75 d (65 %). In the last stage after 85 d of continuous operation the reactor achieved a constant COD removal efficiency of 48 %.The methane production reached constant values around 30 d after the COD removal efficiency was stabilized (0.9, 0.21 and 0.11 g CH_4-COD/gCOD) for a pesticides COD fraction of 20, 30 and 40 % of the TOC, respectively. These may be the reduction of the biodegradable COD available in each stage and the generation of more toxic intermediates. This fact can be also attributed to the bioactivity which can be partially inhibited by the increased pesticides load. Moreover, according to the results obtained in the inhibition test, imidacloprid and dimethoate caused an irreversible inhibition over the methanogenesis which could cause the decrease of the methanogenic biomass activity.

MCPA was not degraded during the first stage, but a removal efficiency of 83 % was achieved in the second stage. This suggests that a long acclimation period is required to get an active MCPA biodegrading biomass. MCPA probably is transformed under anaerobic conditions by means of reductive dechlorination reactions (Puyol et al., 2011). Methanogenic anaerobic sludge has been reported as a very effective biocatalyst of dechlorination (Baczynski et al.,

2010). However, when MCPA feed concentration was increased to 100 mg/L, the removal efficiency decreased to 26 % which indicates the occurrence of inhibition and/or toxicity phenomena.

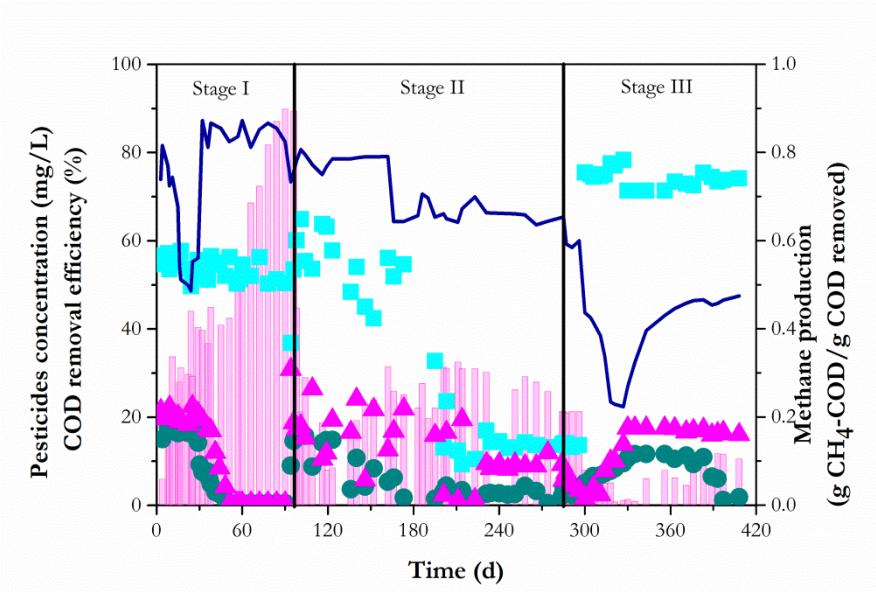

Figure 5.12. COD removal efficiency (continuous line), methane production (bars), and concentration of pesticides: MCPA (squares), imidacloprid (circles), dimethoate (triangles) and cyclohexanone (star) in the effluent during the EGSB reactor operation treating different pesticides loading rates.

In the first stage imidacloprid and dimetoathe needed an acclimation period of 25 and 36 d, respectively, to start their degradation and more than 60 d to achieve an efficiency removal of 98 %. In the second stage the increased of the imidacloprid and dimethoate feed concentration to 29 and 38 mg/L, respectively, caused a severe decrease in the removal efficiency of imidacloprid (86 %) and dimethoate (85 %), being necessary 83 and 141 d for imidacloprid and dimethoate, respectively. Imidacloprid recovered a degradation rate of 95 % after almost 100 d in the last stage while dimethoate removal efficiency frankly decreased down to 68 %. Imidacloprid is biodegraded through the reduction of the nitro group which is a very common transformation reaction in anaerobic environments

(Morgensen et al., 2003). Dimethoate could be degraded following 2 different pathways, either by attacking the alkoxy group (Dauterman et al., 1959), or by demethylation of the methylamide moiety of dimethoate (Lucier and Menzen, 1968).

Cyclohexanone was completely degraded along the experiment, even after every stage initiation, which reinforce the biodegradable character of this compound. This species can be degraded under anaerobic conditions via VFAs formation (Grbic-Galic and Young, 1985; Grbic-Galic and Vogel, 1986).

According to Chin et al. (2005) the system not only was sensitive to the pesticides, also it was sensitive to the concentration of the target compounds due to in each step (when pesticide concentration were increased) the reactor required time to achieve the stationary state as well as the biomass needed an period of adaptation to the variation of the pesticide concentration.

The removal efficiencies of pesticides achieved in the EGSB reactor are fairly higher than those reached in batch test. It is well-known that EGSB reactor enhances the degradation of hardly biodegradable compounds due to in EGSB reactor the recirculation of the effluent keeps diluting the reactor contents (Tsuseef at el., 2013). In addition, the presence of biodegradable compounds added to the synthetic growth medium can also accelerates the biodegradation of persistent molecules by means the activation of enzymes that participate in pollutant degradation (Ortíz et al., 2013). Several examples of co-substrates used to stimulate the degradation of pesticides under anaerobic conditions are sugars and alcohols to favor PCP dechlorination (Puyol et al., 2009), dextrose to improve atrazine biodegradation (Ghosh and Philip, 2004) and starch for removing lindane (Quintero et al., 2006).

Granular structure of EGSB sludge

The morphological structure of the granules was studied and the micrographs of the inoculum and biomass sampled from the EGSB reactor at the end of every stage during the continuous experiment are shown in Figure 5.13. No significant size variations were observed, and granules showed a dense structure up to a TOC pesticides contribution in the feed of 30 %. Higher concentrations led to an increase in holes and crevices on the surface of the granules. Pictures of the cross-section of the granules can be seen in the micrographs depicted in Figure 5.13. It was observed that internal compactness was affected by the presence of pesticides, showing fluffy granules at increasing concentration, where crevices were easily observed. Different structures were observed within the granules, as bamboo-shaped filament and spherical bodies which are commonly associated to the presence of *Methanosaeta* sp. and *Methanosarcina* sp., respectively. *Methanosaeta* sp. predominates in the reactors start-up, when acetate concentration is low, as in the present study (data not shown). However, *Methanosarcina* sp. dominates the environments where the acetate concentration is high (more than 500 mg/L) (Subramanyam et al., 2013). *Methanosaeta* sp. and *Methanosarcina* sp. also were found in the anaerobic treatment of chloronitrobenzenes (Zhu et al., 2015), nitrobenzene, a pesticide precursor (Lin et al., 2013), and organochlorines (Buzzini et al., 2006).

Evolution of the archaeal population of the granular sludge

Figure 5.14 shows the DGGE band patterns for the archaea domain from the anaerobic granular sludge at the startup and after 90 and 285 d of EGSB reactor continuous operation, which corresponds with a pesticide loading rate of 20 and 30 % of the TOC fed. Type and number of archaea band patterns changed during the experiment because of the specialization of the granular sludge to treat pesticides bearing wastewater. Bands were excised, reamplified and sequenced for microbial identification by means of the NCBI and RDP databases (Table 5.3).

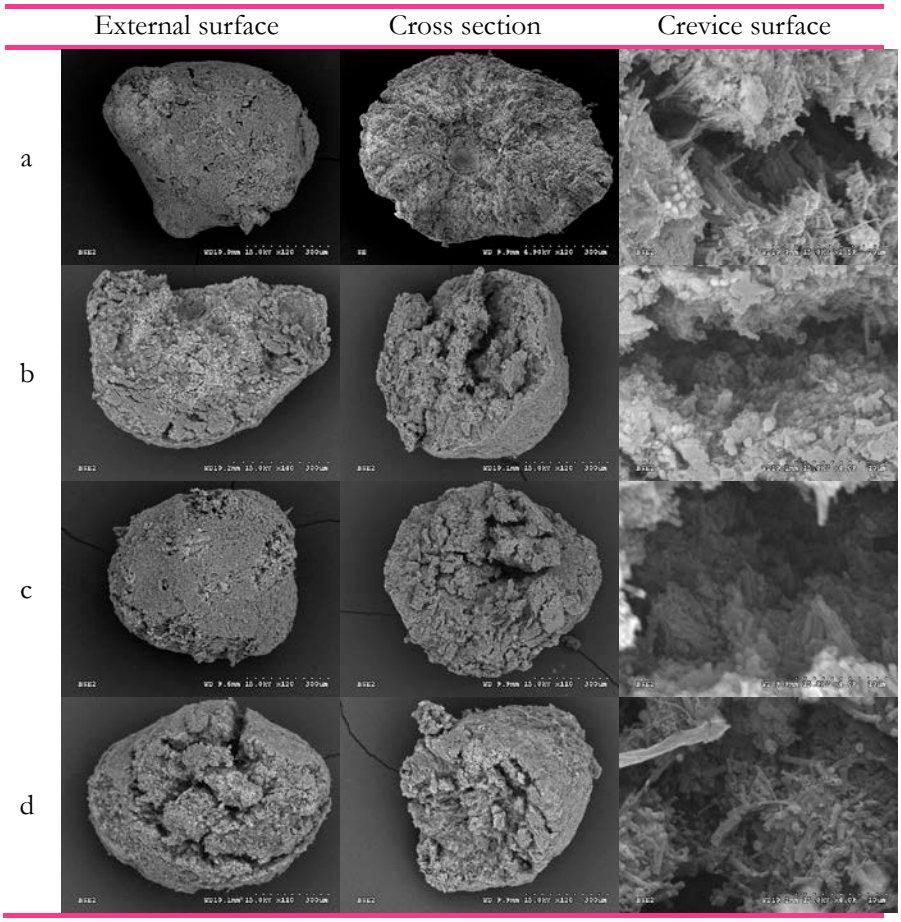

Figure 5.13. SEM micrographs of the granules (external surface, cross section and crevice surface) from the initial inoculum (a) and at the end of stages treating feeds with TOC pesticides fraction of 20 (b), 30 (c) and 40 % (d).

Three identified bands in the sludge iniculum remained during the treatment (A1-5, A9-11 and A15). However the band A14 disappeared during the 20 % TOC phase and appeared again after 285 d. Bands A1, 2, 4, 5, 10 and 11 were identified as *Methanomethylovorans* sp. which uses methanol, methylated amines, dimethyl sulfide and methanethiol for methanogenesis. This obliged methylotrophic archaea are able to form a stable community along with the acetoclastic and hydrogenotrophic methanogens (Lomas et al., 1999). It had been also

identified in oil contaminated groundwater (Watanabe et al., 2002) and in the treatment of wastewater from dyes industries in an anaerobic baffled reactor (Plumb et al., 2001). Band A3 corresponds to the *Methanobacterium* genus which usually uses hydrogen as electron donor, although some species can also oxidize formate, secondary alcohols and carbon monoxide (Garrity and Holt, 2001). *Methanobacterium* is responsible of extracellular polymers production which plays an important role in binding bacteria to form granules (Hulshoff Pol et al., 2004) and possess strong survival and tolerance capabilities (Lin et al., 2013). *Methanobacterium* has been found in the microbial community responsible of nitrobenzene degradation (Lin et al., 2013), atrazine removal in a hybrid UASB reactor (Mullai et al., 2011), and in the treatment of industrial phytopesticide wastewater (Mullai and Sobiya, 2014). New bands of archaea (A6-8) appeared during the first stage in presence of pesticides. Band A6 was not identified but probably corresponds to the genus *Methanobacterium* as band A12. *Methanobacterium formicicum* (A7 and A13) has been identified in the microbial community treating MCPA, imidacloprid and dimethoate, as well as of nitroaromatic compounds (Gorontzy et al., 1993) and 2,4-dichlofophenol (Sponza and Cigal, 2008). Band A8 belongs to *Methanosaeta* and band A14 to *Methanosarcina*, both were identified at pesticides loading rate contributing of 20 % and 30 % of the TOC fed, respectively. *Methanosaeta* prevailed over *Methanosarcina* at low acetate concentration, and *Methanosarcina* domined at high acetate concentration (Smith and Ingram-Smith, 2007). The change in the methanogenic community to *Methanosarcina* sp. could be promoted when dealing with stress conditions and adapting to the new conditions such as highly toxic effects or changes in pesticides inlet composition. *Methanosarcina* are often more tolerant to inhibitors because of their morphology and metabolism, capable of using the acetoclastic and hydrogenotrophic pathways (De Vrieze et al., 2012). *Methanobacterium petrolearium* (A9 and A15) was identified in the phase where pesticides were added due to this archaea was not present in the inoculum. As aforementioned it is a hydrogen utilizing archaea which

needs acetate and yeast extract for its growth (Mori and Harayama, 2011).

Hydrogenotrophic archaea domined the microbiota during the continuous run, highlighting its greater resistance to the presence of toxic compounds. This finding is in accordance with the inhibition test in which the hydrogenotrophic biomass was more robust towards the presence of target pesticides than the acetoclastic community.

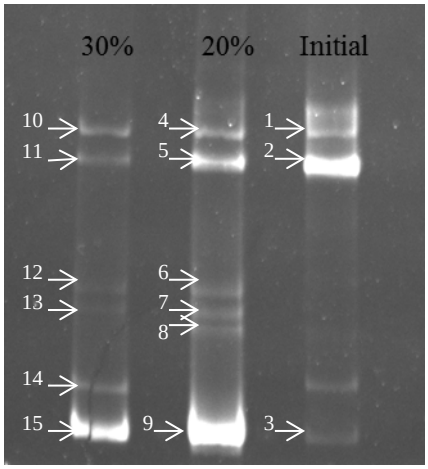

Figure 5.14. DGGE band pattern for archaea domains of the initial anaerobic granular sludge inoculum and during the long-term experiment treating a pesticides TOC fraction of 20 and 30 % in the feed.

5.4. CONCLUSIONS

Dimethoate was successfully removed within the concentration range studied. Two mechanisms for its degradation have been proposed, one start with the attack of the alkoxy group and the second with the demethylation of the methylamine moiety. Imidacloprid degradation occurred by the reduction of the nitro group following a two-stage degradation model. However, MCPA was poorly biodegraded under anaerobic conditions. Binary and tertiary mixtures caused an antagonistic effect over MCPA and dimethoate degradation efficiency, MCPA biodegradation was not improved and dimethoate degradation

was reduced by a 50 %. However, imidacloprid initial removal rate was enhanced by the presence of the other pesticides studied. Insecticides provoked an irreversible inhibition over the acetoclastic archaeas, while only dimethoate was toxic for hydrogenotrophic biomass. This fact corroborates that hydrogenotrophic archaeas are more robust against toxic shocks than the acetoclastic ones. DGGE analysis showed that *Methanobacterium* genus, a hydrogenotrophic archaea, prevailed in the granular biomass during the long-term experiment. The selected pesticides could be successfully biodegraded using and EGSB reactor adding a cosubstrate which promotes the degradation of the toxic compounds. The system showed a high tolerance to pesticides and also cushioned the variations of their concentration.

Table 5.3. Identification of the DGGE bands for archaea present in the sludge granules sampled from the EGSB reactor.

DGGE band	Sequence with higher homology	Similarity (%)	NCBI GenBank access number	RDP taxonomical hierarchy	Ref
1	*Methanomethylovorans* sp.	18 %	KC876048.1	*Methanomethylovorans* (100 %)	Chai et al., 2013
2	*Methanomethylovorans* sp.	18 %	KC876048.1	*Methanomethylovorans* (98 %)	Chai et al., 2013
3	Uncultured archaeon	18 %	AB447830.1	*Methanobacterium* (71 %) *Methanobacteriaceae* (100 %)	UN
4	*Methanomethylovorans* sp.	90 %	KC876048.1	*Methanomethylovorans* (90 %)	Chai et al., 2013
5	Uncultured *Methanomethylovorans* sp.	83 %	KF581805.1	*Methanomethylovorans* (70 %) *Methanosarcinaeae* (80 %)	UN
6	NS				
7	*Methanobacterium formicicum* sp.	99 %	JX042445.1	*Methanobacterium* (90 %)	UN
8	Uncultured *Methanosarcinales* archaeon	99 %	CU916023.1	*Methanosaeta* (100 %)	Riviere et al., 2009

9	*Methanobacterium petrolearium*	99 %	NR_113044.1	*Methanobacterium* (98 %)	Partial sequence
10	Uncultured archaeon	99 %	HQ440110.1	*Methanomethylovorans* (39 %) *Methanosarcinales* (85 %)	Deng et al., 2012
11	*Methanomethylovorans* sp.	99 %	KC876048.1	*Methanomethylovorans* (99 %)	Chai et al., 2013
12	Uncultured archaeon	100 %	AB447830.1	*Methanobacterium* (99 %)	UN
13	*Methanobacterium formicicum*	99 %	JX042445.1	*Methanobacterium* (98 %)	UN
14	Uncultured archaeon	99 %	HQ224858.1	*Methanosarcina* (100 %)	Zhang et al., 2011
15	*Methanobacterium petrolearium*	100 %	NR_113044.1	*Methanobacterium* (100 %)	Partial sequence

Uncultured: not grown on standard media.
UN: Unpublished.
NS: BLAST tool returns Non Significant responses for any query length.

5.5. REFERENCES

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6

ANAEROBIC TREATMENT OF A HIGHLY POLLUTED PESTICIDES-BEARING WASTEWATER UNDER MESOPHILIC AND THERMOPHILIC CONDITIONS

6. ANAEROBIC TREATMENT OF A HIGHLY POLLUTED PESTICIDES-BEARING WASTEWATER UNDER MESOPHILIC AND THERMOPHILIC CONDITIONS

Abstract

The anaerobic treatment of hardly biodegradable wastewater bearing pesticides in an expanded granular sludge bed (EGSB) reactor at mesophilic (35 ºC) and thermophilic (55 ºC) conditions have been studied. Working in these conditions, COD removal efficiencies of 33 and 44 %, respectively, were achieved. The enhancement of the biomass activity in the thermophilic reactor increased the methane production by a 35 %. Around a 96 % of the identified wastewater compounds (pesticides and adjuvants) were not detected in both mesophilic and thermophilic effluents. The microbial popullation dramatically changed with the increase of temperature, although *Methanosaeta* sp. was detected under meso and thermophilic conditions. The toxic effect of pesticides caused a significant inhibition of the methanogenesis, which was critical for the acetoclastic methanogens. In order to improve the mineralization of organic matter an aerobic post-treatment was performed using unacclimated activated sludge. The analysis of the aerobic biodegradability revealed that an anaerobic technology and a subsequent aerobic treatment could be efficiently accomplished, achieving a COD removal efficiency of 62 %.

6.1. INTRODUCTION

The increasing industrial activity has caused that numerous pollutants are released into the environment, which can finally reach water reservoirs. Some of these substances have been declared as priority pollutants in the EU legislation, including several pesticides (Directive 2008/105/EC). Moreover the Drinking Water Directive (98/83/EC) sets a limit of 0.1 μg/L for a single active ingredient of pesticide, and 0.5 μg/L for the sum of all individual active ingredients detected. The major pathways through pesticides may enter into surface and groundwaters are related with the intensive agriculture through runoff and erosion, leaching, drainage (Vymazal and Brezinova, 2015) and by means of discharges from pesticide production plants (Pouran et al., 2015).

The current paradigm of agriculture intensification through pest controls has derived into an increased demand of pesticides. Pesticides industry generates approximately 150 million tons of wastewater annually (Xiong et al., 2011). Wastewater from these sources is characterized by a high heterogeneous nature (Zapata et al., 2010) in terms of a variability of pesticides (herbicides, insecticides, fungicides, rodenticides, nematicides, microbiocides, and plant and insect growth regulators) which contamination levels can be as high as 500 mg/L (Chiron et al., 2000). Its chemical oxygen demand (COD) is very high (23.39–66.30 g/L) but biological oxygen demand (BOD) is relatively low (1.9-7.7) (Chen et al., 2007; Pliego et al., 2012, 2014; Pariente et al., 2013; Xu et al., 2015) . Some of the common physical and chemical wastewater treatment processes including steam-stripping, activated carbon and resin adsorption, chemical oxidation, coagulation/flocculation, hydrolysis, and heavy metals separation (Zhang and Pagilla, 2010).

However, few it has reported on the use of biological systems as a sole technology for the treatment of pesticides bearing wastewaters. An immobilized biomass reactor (IBR), colonized by activated sludge

from a municipal wastewater treatment plant, was used to treat an industrial wastewater resulting from phytopharmaceutical plastic containers washing (Moreira et al., 2012). A significant decay in COD (1662–1960 mgO_2/L) of 46–54 % was observed. Only three (S-metolachlor, alachlor and terbuthylazine) of the 19 pesticides detected in the wastewater, showed a reduction (30–40 %) when their concentration in wastewater were 40.63, 8.74 and 8.51 mg/L of S-metolachlor, alachlor and terbuthylazine, respectively. Aerobic treatment of wastewater from an organic phosphorus pesticide plant was studied by Lin (1990). Pesticide components are tarmaron (44.9 mg/L) and acephate (6.4 mg/L). Aerobic treatment of the wastewater reached COD removal efficiencies of 91 %. After digestion, acephate was undetectable, but the tarmaron concentration was still high in the reactor.

Several works have addressed the biodegradability of pesticides. Under aerobic conditions phenoxyalkanoic acid herbicides as mecoprop (MCPP), dichlorprop, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) can be degraded between 50 and 100 % for initial concentration ranging from 30 to 500 mg/L, using different bioreactors as membrane bioreactor (MBR), fluidized bed bioreactor (FBBR), sequencing batch membrane bioreactor (SB-MBR) and sequencing batch reactor (SBR) (Zipper et al., 1999; Buenrostro-Zagal et al., 2000; Gonzalez et al., 2006; Celis et al., 2008; Sanchis et al., 2013). The biodegradability of nitrochlorinated pesticides (atrazine and diuron) was assessed using an SBR reactor, achieving removal efficiencies from 60 to 95 % when the initial concentration was between 30 and 90 mg/L (Stasinakis et al., 2009; Sanchis et al., 2013). Stasinakis et al. (2009) also have studied the removal of diuron under anoxic conditions, obtaining a removal efficiency of 95 % at starting concentrations among 50–90 mg/L. A biological denitrification system was capable of removing trifluralin (7 mg/L), fenitrothion (7.6 mg/L) and endosulfan (10.9 mg/L), achieving degradation efficiencies up to 95 % (Aslan and Turkman, 2006).

Under strict anaerobic conditions only few studies have reported on the anaerobic biodegradation of pesticides. In aqueous medium under anaerobic environments, chlorpyrifos (1 mg/L) was degraded around a 66 % in 60 d (Tiwari and Guha, 2014). Cyclodiene pesticides (aldrin, isodrin, dieldrin and endrin) at initial concentration between 7–9 mg/L, were dechlorinated by methanogenic granular sludge, achieving removal efficiencies from 59 to 100 % (Baczynski et al., 2004). Ramanand et al. (1993) have reported the biodegradation of the herbicide picloram under methanogenic conditions. More than 85 % of the picloram (340 μM) was degraded in 30 d after a period of acclimation and further reduced to undetectable levels by 100 d. Atrazine removal of 50 % was achieved in wetland sediment and in upflow anaerobic sludge blanket (UASB) reactor using concentrations 5–10 mg/L (Chung et al., 1996, Ghosh et al., 2005). Anaerobic treatment of low-strength wastewater bearing the pesticide pentachlorophenol (PCP) can be carried out in an expanded granular sludge bed (EGSB) reactor operating at 16 mg/L·d of PCP (Lopez et al., 2013).

Anaerobic industrial wastewater treatment is usually used as a pretreatment since anaerobic processes commonly detoxify organics by performing substitutions, as alkylation or dehalogenation. In addition, anaerobic effluents still contain solubilized organic matter which could be appropriated for a subsequent aerobic post treatment (Gray, 2005). The main advantages of the combined anaerobic-aerobic system which are energy production, high treatment efficiency, less sludge disposal, less energy requirements and no volatilization occurs during the aerobic step due to the volatile compounds are degraded in the anaerobic pretreatment have reported by Chang et al. (2009). In this review it had also highlighted the anaerobic–aerobic treatment as an efficient method to treat a huge variety of industrial and municipal wastewater. Nevertheless, a small number of works have reported the combination of different biological systems in order to biodegradate pesticides. Cesar and Ros (2013) used a fixed-bed biodenitrification

reactor connected to an aerobic expanded-bed reactor for the removal of atrazine (0.2–0.43 μg/L) and metolachlor (0.48 μg/L) from groundwater. The two-stage process removed up to 25 % of atrazine and 45 % of metolachlor. Shawaqfeh (2010) assessed the treatment of simulated wastewater containing Vydine (triadimenol) using aerobic and anaerobic reactors and a combination of both. More than 96 % of Vydine (25 mg/L) was removed after an acclimation period of approximately 172 d (aerobic) and 230 d (anaerobic). The combination of anaerobic and aerobic biological processes reduced the retention time, showing an HRT optimum of 24 h for aerobic and 12 h for anaerobic.

The aim of this work is to assess the biodegradability and the inhibitory effect of a highly polluted pesticide wastewater on the methanogenic performance of the granular sludge under anaerobic conditions. Long term-experiment was conducted using an EGSB reactor for evaluating the feasibility of the anaerobic digestion and determining the optimal operating conditions. Moreover, the microbial population during the long-term experiment was studied.

6.2. MATERIALS AND METHODS

Wastewater

Wastewater was collected from a pesticides factory located at Community of Madrid (Spain). Table 6.1 summarizes the average values of the main characteristics of the raw wastewater and the discharge limits for industrial wastewater into the municipal sewer system, according to the 10/1993 Act of the Community of the Madrid (Spain).

Biomass source

Anaerobic granular biomass was collected from a full-scale UASB reactor treating beet sugar wastewater (Valladolid, Spain). The granules had an average diameter of 0.5 mm and a specific methanogenic activity (SMA) of 0.269 (0.006) gCH_4-COD/gVS·d.

Table 6.1. Characteristic of the raw wastewater and the permitted discharge limits (number of samples = 4).

Parameter	Raw wastewater (standard deviation in brackets)	Emission limit value
pH	5.97 (0.8)	6-10
Conductuvity (mS/cm)	1.49 (0.54)	7.5
BOD_5 (g/L)	7.7 (1.99)	1
COD (g/L)	23.39 (1.99)	1.75
Soluble COD (g/L)	15.39 (0.79)	-
TOC (g/L)	8.16 (1.19)	-
TSS (g/L)	6.24 (0.52)	1
VSS (g/L)	6.12 (0.47)	-

Anaerobic biodegradability and methanogenesis inhibition test

Anaerobic batch tests were performed for 30 d inoculating 1.5 g volatile solids (VS)/L of non-adapted granular sludge. The experiments were carried out at 30±1 °C by duplicate, using the Automatic Methane Potential Test System (AMPTS, Bioprocess Control, Sweden) as reported by Garcia-Mancha et al. (2012).

Biodegradability tests were carried out by diluting the raw wastewater to obtain chemical oxygen demand (COD) concentrations ranging from 1.2 to 9.1 g/L. Biodegradability was explained by both soluble COD uptake as well as methane production.

The inhibition of acetoclastic and hydrogenotrophic methanogenesis was studied by using sodium acetate (4 g/L) or sodium formiate (2 g/L). The diluted wastewater was added to both media at COD concentrations of 1.8 (0.05)–12.8 (2.53) gCOD/L.

Tests of volatilization were performed under identical operating conditions as in the biodegradation experiments but in absence of biomass.

Aerobic biodegradability tests

Fast biodegradability of the raw wastewater and the anaerobic effluents was studied using a Liquid–Static–Static (LSS) respirometer (Chica et al., 2007) following the procedure proposed by Polo et al. (2011). The sample (0.9 L) was mixture with activated sludge (3,500 mgVSS/L). Tests were carried out for 24 h at 30 °C.

Experimental set-up for continuous runs

Experiments in continuous mode were carried out using a 5.2 L EGSB reactor with an internal diameter to height ratio of 1:7.2. The reactor was equipped with a gas–liquid–solid separator installed 15 cm below the exit. Details of the apparatus are described elsewhere (Monsalvo et al., 2014). The reactor was operated at an upward flow rate of 2.5 m/h and the hydraulic retention time was 1 d. The EGSB reactor was inoculated with 100 gVS of granular sludge previously activated adding a standard methanogenic medium (2 gCOD/L of a mixture of acetate:propionate:butyrate, 1:1:1 w/w, and 2 g/L of glucose) until a steady stage was reached.

Raw pesticides wastewater was supplemented with 20 mL/L of the following macronutrients solution (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328), $MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and yeast extract (4). This solution was supplemented with 1 mL/L of the subsequent micronutrients solution (μg/L): $FeCl_2 \cdot H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO \cdot 5H_2O$ (162), EDTA (1,000), resazurin (0.2) and sulphuric acid 36 % (1 μL/L). Alkalinity and buffer source was provided by adding 1 g$NaHCO_3$/gCOD.

The reactor was operated under mesophilic (35 ºC) and thermophilic (55 ºC) conditions. First, during the mesophilic stage, the reactor was operated at an OLR of 2 gCOD/L of pesticide wastewater for 76 d. Then, the temperature was increased up to 55 ºC and the methanogenic substrate was used as sole carbon and energy source to acclimate the biomass to thermophilic conditions. After reactor

stabilization at 55 ºC, an OLR of 2 gCOD/L·d of pesticides wastewater without any other organic inputs was applied for 62 d.

Analysis of biomass profile by denaturing gradient gel electrophoresis (DGGE)

The characterization of the biomass profile along the anaerobic processes has been conducted following a standardized DGGE protocol where the bacterial and archeal communities have been targeted independently. Granular sludge was extracted from the EGSB reactors at the beginning of the mesophilic stage and at the end of both mesophilic and thermophilic stages, after 76 and 166 d, respectively. The sludge was resuspended in PBS 1X (pH 7), and cells were disrupted using a BIO101-Savant FP120 cell disrupter (Q-BIOgene, Carlsbad, CA, USA) (six times for 40 s, each at 5.5 cycles/s). DNA extraction, amplification and purification protocols as well as the DGGE procedure, were performed as previously described by Garcia-Mancha et al. (2012). The sequences were compared with those listed in the GenBank nucleotide sequence databases using Chromas 2.0 software. The BLAST search option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database. Determination of the taxonomical hierarchy was performed using the Classifier tool from the Ribosomal Database Project (RDP) web page (http://rdp.cme.msu.edu/index.jsp) for the entire DNA sequences.

Analytical methods

Analyses of COD, total and volatile suspended solids (TSS, VSS) were performed according to the APHA Standard Methods (APHA, 1992).

The identification of species in the raw and treated wastewater was performed by gas chromatography/ion trap mass spectrometry (CP-3800/Saturn 2200, Varian, Santa Clara, CA, USA) equipped with an automatic injector (CP-8200/solid-phase microextraction (SPME)). A 30 m length and 0.25 mm i.d. capillary column (Factor Four VF-5 ms)

was used. The carrier gas (He) flow rate in the GC was 1 mL/min. The SPME was carried out with a fiber cartridge (poly(dimethylsiloxane) red), using adsorption and desorption times of 30 and 5 min, respectively. The sample injection was conducted at 220 °C. The temperature program used in the GC-MS analyses ramped as follows: 40 °C for 5 min, increased to 250 °C at 15 °C/min, held at 250 °C for 10 min, increased to 300 °C at 20 °C/min, and held at 300 °C for 2 min. Compounds identification was assessed using the National Institute of Standards and Technology (NIST) database (http://webbook.nist.gov/chemistry/).

Volatile fatty acids (VFA) were quantified by High-performance liquid chromatography coupled with a refraction index (HPLC/RI) detector (Varian, Agilent Technologies, Santa Clara, CA, USA) using sulfonated polystyrene resin in the protonated form (67H type) as the stationary phase (Varian Metacarb 67H 300 mm–6.5 mm) and sulfuric acid (0.025 N in milliQ water) as the mobile phase at flow rate of 0.6 mL/min. Column temperature was 65 ºC.

6.3. RESULTS AND DISCUSSION

Anaerobic biodegradability and methanogenesis inhibition

Figure 6.1.a shows the time evolution of normalized COD for different starting COD concentrations along the biodegradability test. COD removal efficiency decreased from 49 to 30 % when increased initial COD concentration (1.2–9.1 gCOD/L). This result can be explained due to a less biomass activity when increasing the polluted and toxic load, suggesting inhibition phenomena. This is in accordance with the negligible methane production (lower than 0.005 gCOD-CH_4/gVS·d in all cases) which indicates that methanogenic microorganisms were not active. Gunsalus and Wolfe (1978) have suggested that the final stage in the methanogenic pathway (conversion of CH_3-S-coenzyme M to CH_4) is the step susceptible to pesticide action. The COD decrease could be due to the hydrolysis and acidification stages in anaerobic digestion in which insignificant or

no methane production means good fermentation process (Ince, 1998, Parawira et al., 2008). On the other hand, the COD removal remained constant from around day 18 d probably because of the inhibition of hydrolysis/acidification phase caused by the presence of intermediates generated, according to Viéitez and Ghosh (1999) and Wang and Banks (2003).

To learn about the inhibitory effect of the pesticide wastewater on the methanogenesis, an inhibition test was conducted by adding specific substrates for acetoclastic and hydrogenotrophic methanogenesis. Figure 6.1.b shows the SMA values for both kind of methanogens at different COD concentrations (1.8 (0.05)–12.8 (2.53) gCOD/L). The increase of the COD fraction caused a very different effect on both types of methanogenesis. While the acetoclastic methanogens were almost completely inhibited even at the lowest wastewater concentration tested (1.8 gCOD/L), the hydrogenotrophic methanogens were able to consume the substrate at relatively high specific rates and the complete inhibition was not achieved for the experimental range tested. The hydrogenotrophic archaea only was inhibited by 51 % for the higher wastewater concentration of 12.8 gCOD/L.

Hydrogenotrophic granular biomass was more resistant to the presence of pesticides than the acetoclastic methanogens, which agrees with previous works where the same behaviour was observed for wastewater containing pentachlorophenol (López et al., 2013). When comparing the trophic groups in an AD community, acetoclastic methanogens are generally considered to be the most sensitive to the presence of inhibitors (Astals et al., 2015). Several hypotheses related with the aceticlastic metabolism, which has different behaviour compared to other methanogenic pathways, can explain the high sensitive of this methanogens.

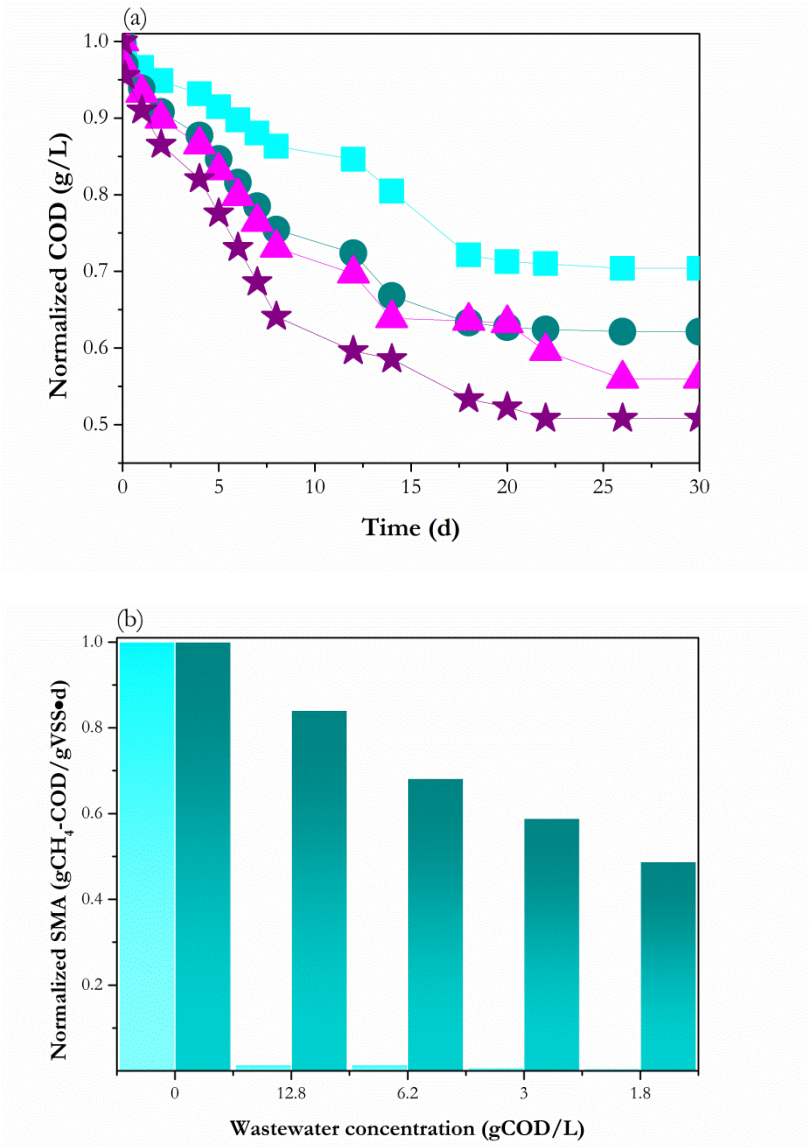

Figure 6.1. Time evolution of (a) normalized COD for different starting concentration of 9.1 (squares), 4.4 (circles), 2.3 (triangles) and 1.2 gCOD/L (stars) during the biodegradability test and (b) normalized SMA in the acetoclastic (cian) and hydrogenotrophic (dark cian) methanogenic inhibition assays at different COD fraction of raw wastewater.

One of them could be the inhibition of different complex and coenzymes involves in the methane production (Chidthaisong and Conrad, 2000; Smith and Ingram-Smith, 2007). Other possible explanation is that some of the acetoclastic (*Methanosarcinales*) lacked a protective envelope around cell wall, which can be easily penetrated by toxicant. On the other hand, some hydrogenotrophic methanogens (*Methanobacteriaceae* and *Methanomicrobiaceae*) can exhibit lower rates of transport of a certain coenzyme into the cell and are likely to be more resistant to toxicants (Xu et al., 2010). Also, the hydrogenotrophic methanogenesis is thermodynamically more favourable than the acetoclastic ones (Deppenmeier and Müller, 2008).

Long term experiment

Mesophilic operation

The performance of the EGSB reactor treating pesticides bearing wastewater is depicted in Figure 6.2. The system operating under 35 ºC needed a period of acclimation of 12 d to start removing COD. The COD removal efficiency increase during the treatment achieving values of 33 % which is maintained along the operation under mesophilic conditions. Along this mesophilic phase, methane yield was progressively improving until reaching efficiencies around 34 %. Also, the concentration of VFAs was followed. Acetate concentration was continuously decreasing during the mesophilic phase. However, its concentration in the effluent was still high, showing the same inhibition phenomena than the observed in the inhibition test. On the other hand, butyrate and propionate were accumulated in the first 30 d of operation, from this day propionate and butyrate concentration decreased by an 85 and 65 %, respectively. This result can be possible due to an effectively H_2 removal by methanogens (van Lier et al., 2008).

Thermophilic operation

The system was tested in thermophilic conditions by increasing the temperature to 55 °C. However, due to the lack of thermophilic

inocula (which are responsible for methane production in thermophilic conditions), it was necessary to transform mesophilic sludge to thermophilic ones. Thus, methanogenic substrate was used as organic source at an OLR of 2 gCOD/L·d (day 76), promoting the metabolic adaptation of the biomass. During this period, the biomass lost completely their activity according with the COD removal efficiency and methane production near zero. Nevertheless the system began its recover quickly (day 81) increasing drastically the COD removal. On the other hand, the concentration of VFAs decreased below detection limits on 94th d, which was in accordance with the change of CH_4 production, suggesting that the selection of the thermophilic microorganisms had taken place. Similar finding were obtained by Tian et al. (2015). Once the thermophilic biomass was established and the reactor started its recovery, the methanogenic medium was not used as influent anymore and the pesticide wastewater was used for feeding the reactor at an OLR of 2 gCOD/L·d (day 104).

Under thermophilic conditions (104th–166th) COD removal efficiency around 44 % was reached after the stabilization of the reactor. Methane production efficiency between 44–46 % was achieved operating at 55 ºC. The increase of temperature allowed higher efficiency in the degradation of organic matter and higher biogas production than the wastewater treatment at mesophilic temperatures according to Leven et al. (2007) and Bassani et al. (2015). The operating temperature is a fundamental variable affecting reactor performance, because of improved hydrolysis rates and methane yields due to favourable kinetics at higher temperatures (Kim et al., 2002; Bocher et al., 2008). Higher hydrolysis rates implied more soluble compounds converted to CH_4, improving the methanogenesis process. The enhancement of the hydrolysis rate also could be attributing to an abiotic process since the reaction rates increase with increasing temperature by the Arrhenius equation. Acetate and butyrate concentration increased during the first 26 d of operation, but from there the concentration decreased, showing a slightly acclimation

to the pesticide wastewater. Nevertheless, acetate was no completely removed, revealing an inhibition over acetoclastic methanogens. During the thermophilic treatment the concentration of acetate is lower than in the mesophilic treatment, likely because of the minimization of toxic effect (van Lier., 1997) or the disappearance of the toxic compound due to the enhancement of the degradation rate of refractory compounds (Wang et al., 2011).

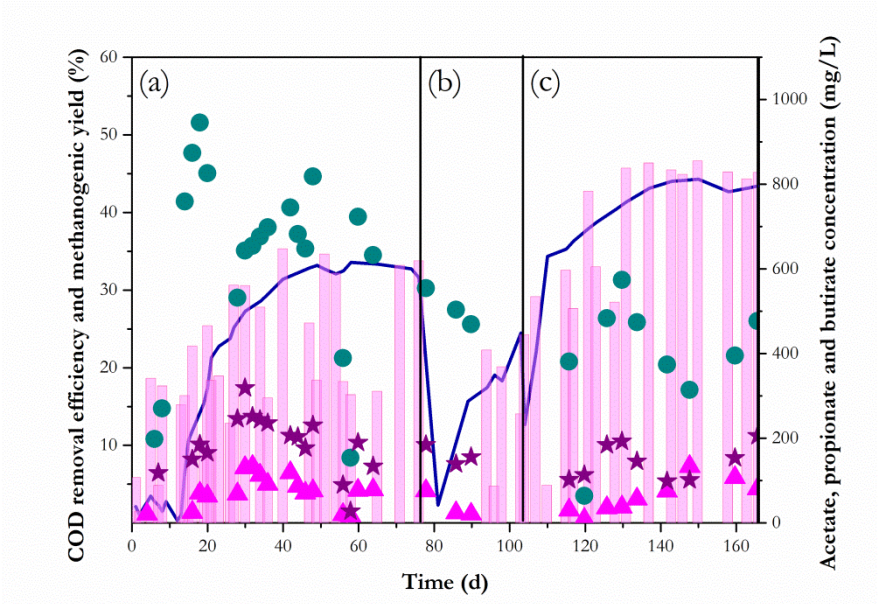

Figure 6.2. Time course of COD removal (line) and methane production (bars) efficiencies and acetate (circles), propionate (triangles) and butyrate (stars) along continuous operation under mesophilic conditions (a), under thermophilic conditions using methanogenic medium as sole carbon source (b) and at 55 ºC feeding the pesticide bearing wastewater (c).

At 55 ºC, propionate concentration was even slightly lower due to thermophilic conditions can favour hydrogenotrophic methanogens (Demirel and Scherer, 2008). Anyway, propionate and butyrate concentrations were no so high due to an efficient metabolism of it during the long term experiment. This is thermodynamically possible only at low H_2 concentrations which were achieved by the presence of

active hydrogenotrophic biomass which was more resistant to this wastewater than the acetoclastic microorganisms.

These findings can be supported by the DGGE band patterns for the archaea and bacteria domains from the anaerobic granular sludge during EGSB reactor continuous operation at 35 and 55 ºC (Figure 6.3). This picture reveals the specialization of biomass occurred during the treatment of pesticides bearing wastewater due to the disappearance of some bands when the operating conditions were changed. Bands were excised, reamplified and sequenced for microbial identification by means of the NCBI and RDP databases (Table 6.2).

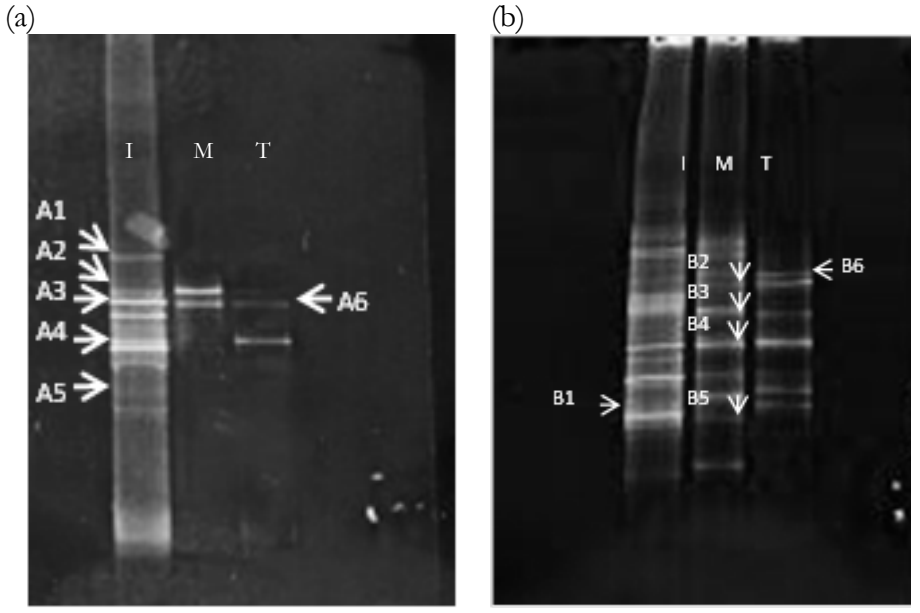

Figure 6.3. DGGE pattern of bands for initial (I), mesophilic (M) and thermophilic (T) (a) archaea and (b) bacteria.

A clear reduction of the archaea and bacteria diversity in the granular sludge took place when temperature was increased from mesophilic to thermophilic range. It has been demonstrated that methanogenic diversity in plants operating at thermophilic conditions are lower than the mesophilic ones (Leven et al., 2007). *Methanosaeta* sp. was presented in seed sludge (A1-A3), they are determining in the

development of the granules, also they usually are the dominant group in the seed sludge when the acetate and VFAs concentrations are low, indicating that the reactor is stable (Mc Hugh et al., 2003; Karakashev et al., 2005). The inoculum proceeds from an UASB reactor feed with a methanogenic medium, due to that it achieved great efficiencies and the acetate and VFAs concentration remained low within the UASB reactor. Band A4 was identified as *Methanobacterium formicicum*, also it was found in a EGSB reactor which treated dairy wastewater (4 gCOD/L) at 37 ºC (Bialeket al., 2011). Band A5 was identified as an archaea from the order *Methanobacteriales*, frequently detected in anaerobic digesters (Mc Hugh et al., 2003). At thermophilic range it only was identified one archaea (A6), *Methanosaeta* sp., which are capable to catabolizing acetate to methane under meso and thermophilic conditions (van Lier, 1996). In the thermophilic lane in the DGGE band patterns it can be highlighting the presence of one band which can be the same as the band A4, *Methanobacterium formicicum* or belong to the same order (*Methanobacteriales*). These results suggested that a temperature increase resulted in a shift in the methanogenic population. *Methanosaeta* sp. was less abundant during the mesophilic treatment due to the insufficient amount of acetoclastic methanogens or due to their inhibition. Under thermophilic conditions these acetoclastic archaea were displace by the order *Methanobacteriales* which contains one extremely thermophilic and one hyperthermophilic genus (Liu and Whitman, 2008).

Bacteria domain retains only 3 bands along the treatment under meso and thermophilic temperatures. Only some bands could be identified. The inoculum includes phylum *Chloroflexi* (B1), which also remain in the sludge after the mesophilic treatment (B5). These bacterias were abundant in anaerobic digesters and can decomposed carbohydrates via fermentation (Narihio et al., 2012). Operating at 35 ºC it was found bacteria correspond to phylums *Firmicutes* (B2) and *Proteobacteria* (*Syntrophobacter* sp., B3). Both are propionate degrading bacteria which can be found in UASB systems (Narihio et al., 2012). Bands B4 y B6

corresponds with a hydrolic and acidogenic bacteria (*B. Subtilis* and *Clostridium* sp., respectively), which also were found in a UASB reactor operating under meso and thermophilic conditions (Khemkhao et al., 2012). It is known that these bacteria accelerate the growth of granules by excreting extracellular polymeric substances (Fukuzaki et al., 1995). It can be highlighted than with the temperature increase hydrolytic bacteria (B4 and B6) remained in the sludge, while fermentative bacteria (B1) disappeared. This result was in accordance to the higher hydrolytic activity showed in the reactor at 55 ºC. On the other hand, in agreement with the low propionate concentration found in the effluent, propionate degrading bacteria (B2 and B3) can be detected under both mesophilic and thermophilic conditions. This result suggests that a possible pathway for the degradation of organic compounds present in the pesticide wastewater could be through the formation of propionate.

Pesticide removal

Table 6.3 summarized the composition of the wastewater from pesticides manufacture detected by GC-MS and the peak area reduction after the treatment under meso and thermophilic conditions. As it is shown in the chromatograms for the initial and treated effluent under mesophilic conditions, most of the starting compounds were not detected in the resulting effluent after 76 d of continuous operation (Figure 6.4). However, herbicides as pendimethalin, tert-butilazine, isoproturon, linuron and metolachlor were detected in the effluent.

Chlorinated compounds (tert-butilazine, linuron, metolachlor, MCPA, MCPP, oxifluorfen and propiconazol) probably are transformed under anaerobic conditions by means of reductive dechlorination reactions (Puyol et al., 2011). Baczynski et al. (2010) has reported that methanogenic anaerobic sludge is a very effective biocatalyst of dechlorination, including some chlorinated pesticides. Terzbutylazine and propiconazole are also nitrogen-containing pesticides.

Table 6.2. Identification of DGGE bands for archaea (A) and bacteria (B) present in sludge granules from the EGSB reactor.

DGGE band	Sequence with higher homology	Similarity (%)	NCBI GenBank access number	RDP taxonomical hierarchy	Ref
A1	uncultured *Methanosaeta* sp.	100	HQ336501.1	*Methanosaeta* sp. (95 %)	U
A2	uncultured *Methanosaeta* sp.	99	HQ336501.1	*Methanosaeta* sp. (92 %)	U
A3	uncultured *Methanosaeta* sp.	99	HQ336501.1	*Methanosaeta* sp. (100 %)	U
A4	*Methanobacterium formicicum*	99	JX042445.1	*Methanobacterium* sp. (100 %)	U
A5	uncultured *Methanobacteriales* archaeon	99	KF198736.1	Methanobacteriales (99 %)	U
A6	uncultured *Methanosaeta* sp.	100	HQ336501.1	*Methanosaeta* sp. (97 %)	U
B1	uncultured bacterium	100	JX100396.1	Bacteria (100 %) *Chloroflexi* (39 %)	Ban et al., 2013
B2	uncultured bacterium	94	AB266992.1	Bacteria (100 %) *Firmicutes*(26 %)	Narihiro et al., 2009
B3	uncultured bacterium	100	JX100401.1	*Syntrophobacter* sp. (97 %)	Ban et al., 2013
B4	*Bacillus subtilis*	99	KF577868.1	Bacteria (100 %) *Bacillus* sp. (52 %)	U

B5	uncultured bacterium	100	JX100396.1	Bacteria (100 %) *Chloroflexi* (59 %)	Ban et al., 2013
B6	uncultured bacterium	99	FR687174.1	*Clostridium III* sp. (92 %)	Liu et al., 2011

U: Unpublished.
Uncultured: not grown on standard media

Their degradation probably involves hydrolytic dechlorination, dealkylation, and the cleavage of C–N in the cyclic ring, as describe Zhang et al. (2004) for atrazine. Organophosphate pesticides (fenamiphos) can be biodegraded by the hydrolysis of phosphate esters, catalyzed by esterase. An esterase hydrolyzed the P–O–C linkage in organophosphate pesticide subsequent to a nitro reduction (Zhang et al., 2004). Other examples of reductive reactions of importance for pesticides are sulfoxide reduction (ethofumesate) and nitro group reduction (pendimethalin and oxifluorfen). The reduction of the nitro group to an amine is a very common transformation reaction in anaerobic environments (Morgensen et al., 2003). Isoproturon degradation pathway under anaerobic condition may be involved a hydrolytic cleavage or reductive cleavage (Drzyzga, 2003) and reductive deamination (Travkin et al., 2002). Mancozeb suffered and hydrolysis to obtained ethyleneurea which was further degraded to 2- imidazoline and other unknown compounds under anaerobic conditions (Xu, 2000).

Figure 6.5 shows the GC-MS chromatograms obtained for the initial and treated effluent under thermophilic conditions whose composition and peak area reduction is detailed in Table 6.3. As in the case of the mesophilic operation, most of these compounds were not detected in the resulting effluent after 62 d of EGSB reactor operation (Figure 6.5.b). Pendimethalin, tert-butilazine, chlorpyrifos, metolachlor, MCPA and oxifluorfen were detected in the effluent. As aforementioned under anaerobic conditions the common reactions to removal pesticides include dehalogenation, hydrolysis, nitro reduction and sulfoxide reduction. In anaerobic environments, chlorpyrifos was directly hydrolyzed to 3,5,6-trichloro-2-pyridinol and released diethylthiophosphate (Tiwari and Guha, 2014). In the degradation of imidacloprid, the nitro group is reduce to desnitro/guanidine and urea metabolites (Pandey et al., 2009) followed by imidazoline ring opening (Ding et al., 2011). It is expected that tert-buconazole follows the same biodegradation pathway as propiconazole.

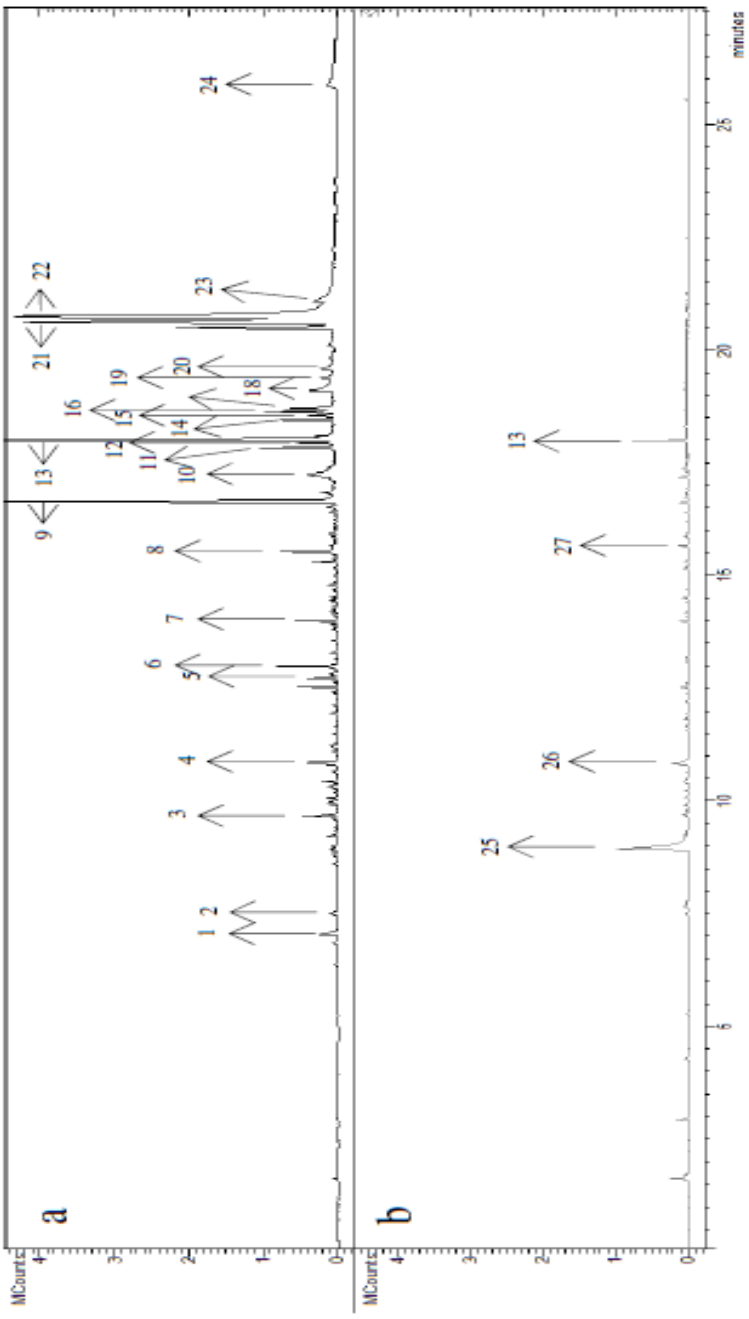

Figure 6.4. Representative GC-MS chromatograph of mesophilic EGSB (a) influent and (b) effluent.

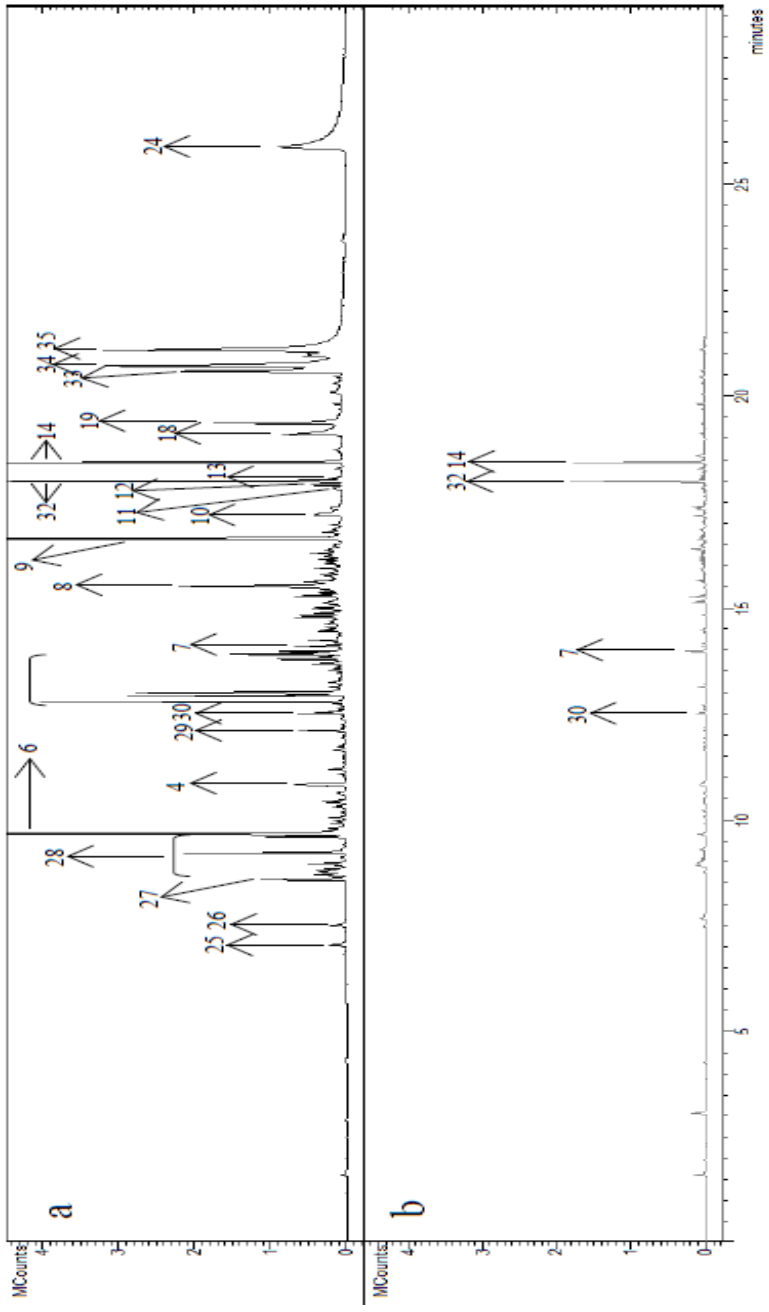

Figure 6.5. Representative GC/MS chromatograph of thermophilic EGSB (a) influent and (b) effluent.

Table 6.3. Removal efficiencies for the compounds identified by GC-MS.

Peak	Compound	Type	Removal efficiency (%) at 35 ºC	Removal efficiency (%) at 55 ºC
1	Xilene	Solvent	>99	ND
2	Dimethylbenzene	Solvent	>99	ND
3	2-ethyl-1-hexanol	Solvent	97	ND
4	1,2,3,4-tetramethylbenzene	Solvent	>99	>99
5	Indole	Pesticide precursor	>99	ND
6	Oxirane [((2-ethylhexyl)oxy)methyl]	Solvent	>99	>99
7	Pendimethanlin	Herbicide	79	53
8	N,N-dimethyldecanamide	Emulsifier, solvent, and co-solvent	>99	>99
9	tert-butylazine	Herbicide	99	99
10	Phtalate	Solvent	>99	88
11	Isoproturon	Herbicide	97	>99
12	Linuron	Herbicide	99	>99
13	Metolachlor	Herbicide	98	75
14	MCPA	Herbicide	>99	74
15	Ethofumesate	Herbicide	>99	ND
16	MCPP	Herbicide	>99	ND
17	5-(2-propenil)-1,2-benzodioxale	Precursor insecticide synthesis	>99	ND

18	Fenamiphos	Insecticide	>99	>99
19	Oxifluorfen	Herbicide	>99	99
20	Mancoceb	Fungicide	>99	ND
21	Inert	Inert	>99	ND
22	Propiconazole	Fungicide	>99	ND
23	Surfactant	Surfactant	>99	ND
24	1,2,4-trichlorobenzene	Intermediate in herbicide production	>99	>99
25	o-xilene	Solvent	ND	>99
26	p-xilene	Solvent	ND	>99
27	Ethylhexenal	Solvent	ND	>99
28	Trimethylbenzene	Solvent	ND	>99
29	Fluorene	Pesticide production	ND	94
30	Dispersant	Dispersant	ND	67
31	Methylnaftalene	Solvent	ND	>99
32	Chlorpyrifos	Insecticide	ND	77
33	Imidacloprid	Insecticide	ND	>99
34	Inert	Inert	ND	>99
35	Tert-buconazole	Fungicide	ND	>99

Pesticides removal efficiency was similar under both meso and thermophilic conditions. It is believe in general that the thermophilic bacteria have similar metabolism as their mesophilic colleagues, in terms of substrate range and end product formation (Duran and Speece 1997).

Aerobic biodegradability

In order to improve the mineralization of organic matter a subsequent aerobic treatment was evaluated. For this purpose aerobic biodegradability assays of the EGSB effluents were evaluated and compared with the biodegradability of the raw wastewater.

Figure 6.6 shows COD removal efficiencies obtained for the systems studied. Raw wastewater showed a biodegradability of 37 %. This result indicated that an aerobic technology, used as sole biological treatment of this pesticides bearing wastewater, is not a feasible solution. This assumption can also be applied to the anaerobic technology which achieved a COD removal of 33 and 44 % under mesophilic and thermophilic conditions, respectively. However, the combination of both anaerobic and aerobic technologies could improve the efficiency of the treatment, making it a feasible option. The biodegradability of the meso and thermophilic effluents reached COD removal efficiencies around 26 and 18 %, respectively. This indicates that the most biodegradable organic compounds were degraded in the anaerobic treatment, especially in the thermophilic process. Nevertheless, the results obtained in the biodegradability test of the EGSB effluents were within the range of values (below 10 to 22 %) achieved when an aerobic system was used as a post-treatment for recalcitrant/toxic wastewater (Deng et al., 2006; Zhang et al., 2008; Koupaie et al., 2011). In this study the proposed system improved the mineralization or organic matter by 62 % combining anaerobic high-rate technologies with a post aerobic treatment.

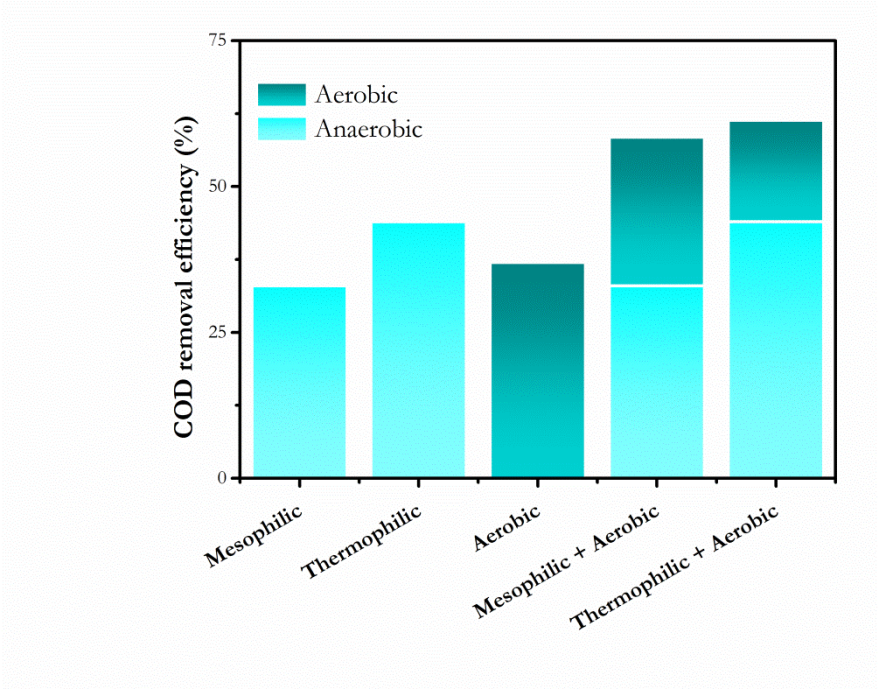

Figure 6.6. COD removal efficiencies achived by mesophilic, thermophilic and aerobic reactors and their combined systems.

6.4. CONCLUSIONS

Anaerobic treatment of a highly polluted pesticides-bearing wastewater using EGSB reactors under meso and thermophilic conditions allows the removal of most pesticides contained in this wastewater from pesticide manufacturing. Under thermophilic conditions an improvement of the biodegradability of the pesticides-bearing wastewater was achieved due to the enhancement of hydrolysis rates with increasing the temperature. This finding implied the existence of more soluble compounds which can be converted to CH_4, improving the efficiency of the methanogenic process. Hydrogenotrophic methanogens showed to be more resistant and consequently more active than acetoclastic archaea. This result is in accordance with the treatment in the EGSB reactor which showed an efficient propionate and butyrate metabolism due to low H_2

concentrations. Finally, the combination of mesophilic or thermophilic anaerobic treatment followed by an aerobic reactor led to COD removal efficiencies significantly higher than those achieved by anaerobic or aerobic systems as sole treatment technology.

6.5. REFERENCES

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7

ANAEROBIC TREATMENT OF WASTEWATER FROM USED INDUSTRIAL OIL RECOVERY

Garcia-Mancha, N., Puyol, D., Monsalvo, V. M., Rajhi, H., Mohedano, A. F., & Rodriguez, J. J. (2012). Anaerobic treatment of wastewater from used industrial oil recovery. *Journal of Chemical Technology and Biotechnology*, *87*(9), 1320-1328

7. ANAEROBIC TREATMENT OF WASTEWATER FROM USED INDUSTRIAL OIL RECOVERY

Abstract

This work is focused on the anaerobic biodegradation of wastewater from used industrial oils (UIO) recovery using a bench-scale expanded granular sludge bed reactor (EGSB) at room temperature. Biodegradability tests showed that this wastewater can be partially biodegraded under anaerobic conditions at mesophilic temperature. Low concentrations of wastewater caused an incremented specific activity of the acetoclastic and the hydrogenotrophic methanogens. Anaerobic biodegradation at room temperature is feasible at organic loading rates (OLR) lower than 5.5 gCOD/L·d. A further increase of the OLR to around 10 gCOD/L·d had a detrimental effect on the system performance, making it necessary to work at mesophilic conditions. Anaerobic treatment using an EGSB reactor is a feasible option for treating UIO wastewater. Long-term treatment caused a specialization of the granular sludge, modifying substantially its microbial composition. Methane production was even stimulated by the addition of UIO wastewater at low concentrations.

7.1. INTRODUCTION

Huge quantities of industrial oil are consumed as a result of their extensive application (more than 310 kt consumed in Spain in 2009) in a broad range of industrial processes. Once used, the resulting oils are considered toxic and hazardous showing fairly low chemical or biological degradability. The amount of these residues has been estimated at around 90 kt in Spain (2009). The used industrial oils (UIO) contain hazardous and toxic compounds, e.g. naphthalene, benzene derivatives and toluene, whose presence in the environment can cause severe damage. The waste hierarchy postulated in the 2008/98/EC European Directive includes valorization as one

preferential strategy for waste management. In Spain, recycling of 65 % of UIO has been postulated as an objective (Spain, Real Decreto 679/2006). Although it is still early to assess the impact of the new regulations, it is expected that the legal framework will reinforce oil recycling and regeneration objectives that will result in additional volumes of aqueous off-streams from these operations, usually including different stages of metals precipitation, extraction and distillation (Boughton and Horvath, 2004). These effluents are characterized by high contents of organic solvents, hydrocarbons and suspended solids (SS), relatively high viscosity and poor biodegradability, making their treatment by conventional biological systems difficult. Nevertheless, biological techniques developed in the last two decades have shown their potential to deal with a broad range of industrial wastewater. In particular, anaerobic systems are an attractive option for the treatment of high-strength wastewater with an associated potential for methane generation (Chan et al., 2009). A first approach to the anaerobic treatment by a two-step process of a synthetic wastewater simulating the effluents from UIO recycling operations showed COD removal efficiencies higher than 83 % (Alimahmoodi and Mulligan, 2011). However, application to real wastewater has not been reported so far.

Among the anaerobic systems, the upflow anaerobic sludge blanket (UASB) reactor is the most widely applied for industrial wastewater. However, the so-called expanded granular sludge bed (EGSB) reactor is a promising alternative in which the height to diameter ratio and the external recirculation rate are increased, improving the mixing and contact between wastewater and biomass (Puyol et al., 2009). The viability of this system has been previously demonstrated in full-scale applications treating real wastewaters (Seghezzo et al., 1998). The height to diameter ratio plays an important role in the operation of EGSB reactors, and usually varies between 7 and 90 depending on the characteristics of the wastewater and the dimensions of the reactor (Zhang et al., 2008; Fang et al., 2011). Thus, EGSB systems have been

reported to be adequate for dealing with hardly biodegradable wastewater, and to dampen the inhibition of the microbial activity caused by the presence of hazardous pollutants. These inhibition phenomena vary widely depending on the anaerobic inocula, wastewater composition, and working conditions. Some compounds commonly found in off-streams from UIO recovery, such as ammonia, sulphide, light metal ions, heavy metals, and organics, including alkil benzenes, phenol, alkil phenols, alkanes and alcohols have been reported to be inhibitors of anaerobic digestion (Lin and Chen, 1999; O'Flaherty et al., 1999; Calli et al., 2005; Chen et al., 2008).

The identification of those microorganisms present in bacterial communities capable of degrading the organics present in this type of wastewater is of great interest for future industrial applications. Molecular biology techniques are useful to gain insights to the phylogenetic characterization of the microorganisms capable of removing chemicals from industrial wastewater. Some molecular techniques, like cloning and sequencing of extracted DNA and RNA, can provide exhaustive microbiological information. Among others, denaturing gradient gel electrophoresis (DGGE) is becoming routinely applied to microbial ecological studies to follow changes in the microbial population during the operation of anaerobic reactors (Díaz et al., 2006).

The aim of this work is to assess the anaerobic biological treatment of wastewater resulting from the regeneration process of UIO, determining the optimal operating conditions of an EGSB reactor. Biodegradability and the toxic effect of this wastewater on the methanogenic performance of the granular sludge under anaerobic conditions have also been evaluated. In addition, DGGE and sequencing of particular bands have been used to evaluate the evolution of the microbial population during the long-term experiment.

7.2. EXPERIMENTAL

Biomass source

Anaerobic granular biomass was collected from a full-scale UASB reactor treating sugar-beet wastewater (Valladolid, Spain). The granules had an average diameter of 0.5 mm and a specific methanogenic activity (SMA) of 0.46 gCH_4-COD/gVS·d.

Wastewater composition

Wastewater was collected from a UIO recovery plant (Madrid, Spain). The main characteristics of the UIO wastewater (10 samples tested) were: 29±5 $gBOD_5$/L, 107±47 gtotalCOD/L, 94±38 gsolubleCOD/L, 2.87±1.07 gTSS/L, 2.67±1.12 gVSS/L and pH 10.5±0.8. Ethylene-glycol was identified as the most abundant chemical with a concentration of around 27.6±0.9 g/L.

SMA determination

Specific methanogenic activity (SMA) was measured using the Automatic Methane Potential Test System (AMPTS) developed by Bioprocess Control AB (Lund, Sweden). The AMPTS follows the same principles as the conventional methane potential test, thus making the results comparable with standard methods. Methane released from the digestion bottles is analyzed using a wet gas-flow measuring system with a multi-flow cell arrangement. This measuring device works according to the principle of liquid displacement and can monitor an ultra-low gas flow, where a digital pulse is generated when a defined volume of gas flows through the system. It only registers methane flow, since other gas components, such as CO_2 and H_2S, are removed by an alkaline solution. A data acquisition system is incorporated (Jiu, 2010). SMA values were calculated by the Roediger model (Edeline, 1980) according to a previous work (Puyol et al., 2009).

Biodegradability tests and methanogenesis stimulation experiments

Biodegradability tests were performed for 32 d using a non adapted sludge by adding wastewater diluted at different ratios ranging from 6.25 to 50 % (v/v), which corresponds to COD values from 4 to 32 g/L. Stimulation of acetoclastic and hydrogenotrophic methanogenesis was carried out by activating the anaerobic sludge with acetate (4 gCH_3COONa/L) or formiate (2 g$HCOONa$/L), respectively, added to a standard methanogenic medium containing the following macronutrients (mg/L): NH_4Cl_2 (280), K_2HPO_4 (250), KH_2PO_4 (328),$MgSO_4 \cdot 2H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (10) and yeast extract (4). This medium was supplemented with 1 mL/L of a trace elements solution, reaching (μg/L): $FeCl_2$ $4H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot 2H_2O$ (38), $MnCl_2 \cdot 4H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (92), Na_2SeO $5H_2O$ (162), EDTA (1,000), resarzurin (200), H_2SO_4 36 % (1 μL/L). Buffer and alkalinity source was incorporated by adding 1 g$NaHCO_3$/gCOD. The UIO wastewater was added to both media at COD concentrations ranging from 0.125 to 2 g/L. The biodegradability tests and methanogenic stimulation experiments were performed at 30±1 ◦C in duplicate, using the AMPTS.

The contribution of adsorption was evaluated in biomass samples after extraction with Soxhelt following the US-EPA 8041 method. Tests of volatilization were performed under identical operating conditions to those in the biodegradation experiments but in the absence of biomass. The results reported are the average values from duplicate runs, the standard errors being always lower than 10 %.

Experimental setup for long-term experiment

Experiments in continuous mode were carried out using a 5.2 L EGSB reactor with an internal diameter to height ratio of 1:7.2. The reactor was equipped with a gas–liquid–solid separator installed 15 cm below the exit. Wastewater was continuously fed, entering at the bottom of the reactor with recirculation, and the effluent was

withdrawn from the top. CO_2 was removed from biogas using a Mariotte flask trap with 4 mol/L NaOH solution, and CH_4 was measured with a wet gasometer (Schlumberger, Germany) (Puyol et al., 2011). The reactor was operated at an upward flow rate of 2.5 m/h and room temperature (17–21 ºC) for 80 d. The EGSB reactor was inoculated with 100 gVS/L of granular sludge previously activated with a standard methanogenic medium for 30 d until high-activity stable performance was achieved. Macro and micronutrients, as well as $NaHCO_3$ (buffer and alkalinity source) to neutralize the influent were supplemented as detailed above. Organic loading rate (OLR) was varied between 0.5 and 10.5 gCOD/L d during the course of the experiment.

DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE)

Granular sludge was resuspended in PBS, and cells were disrupted using a BIO101-Savant FP120 cell disrupter (Q-BIOgene, Carlsbad, CA, USA) (six times for 40 s, each at 5.5 cycles/s). DNA was extracted using the FastDNA kit for soil (Q-BIOgene, Carlsbad, CA, USA). A fragment of the 16S rRNA gene was amplified by PCR with primer pairs 341(GC)-907R for Bacteria and 622(GC)-1492R for archaea at annealing temperatures of 52 and 42 ºC, respectively (Chan et al., 2001). The amplification reaction was performed according to the Taq DNA polymerase protocol (Promega, Madison, WIS, USA). The PCR conditions were as follows: 94 ºC for 10 min; 30 cycles at 94 ºC for 1 min, 72 ºC for 3 min; and 72 ºC for 10 min. The PCR products were analyzed using a D-Code Universal system (Bio- Rad, Hercules, CA, USA). An acrylamide solution with 6 % (w/v) of porosity was used to cast a gel with denaturing gradients of urea/formamide ranging from 30 to 60 % (100 % = 7 mol/L urea/40 % v/v formamide). Electrophoresis was conducted in 1X TAE buffer solution at 200 V and 60 ºC for 5 h. Bands detected by fluorescence using a UV transilluminator were excised and reamplified for sequencing. The sequences were automatically analyzed on an ABI

model 377 sequencer (Applied Biosystems, Carlsbad, CA, USA) and were thereafter corrected manually. The sequences were compared with those listed in the GenBank nucleotide sequence databases using Chromas 2.0 software. The BLAST search option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database. Determination of the taxonomical hierarchy was performed using the Classifier tool from the Ribosomal Database Project (RDP) web page (http://rdp.cme.msu.edu/index.jsp) for the entire DNA sequences.

Analytical methods

Analyses of chemical oxygen demand (COD) and total and volatile suspended solids (TSS and VSS) were performed according to the APHA Standard Methods. The identification of species in the influent and treated effluents was performed by gas chromatography/ion trapmass spectrometry (GC/MS, CP-3800/Saturn 2200, Varian, Santa Clara, CA, USA) with an autosampler injector (CP-8200, Varian, Agilent Technologies, Santa Clara, CA, USA) and solid-phase microextraction (Carbowax/Divinylbenzene, Yellow- Green) (Puyol et al., 2011). Ethlylene-glycol, propylene-glycol, ethanol and volatile fatty acids (VFA) were quantified by HPLC coupled with a refraction index (HPLC/RI) detector (Varian, Agilent Technologies, Santa Clara, CA, USA) using sulfonated polystyrene resin in the protonated form (67H type) as the stationary phase (Varian Metacarb 67H 300–6.5 mm) (Puyol et al., 2011).

7.3. RESULTS AND DISCUSSION

Anaerobic biodegradability

Figure 7.1.a shows the time-evolution of COD and the cumulative methane production during the biodegradability tests. The specific COD consumption rate decreased from 0.34 to 0.11 gCOD/gVS·d as the COD of the fed stream was increased from 4 to 32 gCOD/L. Simultaneously, the specific methane production rate decreased from

0.23 gCH_4-COD/gVS·d to almost zero under those same conditions. These results suggest the occurrence of inhibitory phenomena, since the activity values decreased when increasing the organic load of the UIO wastewater. Effects of adsorption or volatilization were negligible, so the COD removal can be attributed exclusively to biological degradation. A methane production of 1.15 gCH_4-COD was obtained at an UIO wastewater COD of 4 g/L, reaching a methanogenic potential of around 65 %. In all cases, the methane production was lower than the corresponding theoretical value which supports the occurrence of inhibition. To learn more about the inhibition phenomena, the time-evolution of the main metabolites was analyzed. The time-evolution of ethylene-glycol and acetate is plotted in Figure 7.1.b. As can be seen, ethylene-glycol was oxidized to acetate before methanization, which is in agreement with previous works (Veltman et al., 1998; Staples et al., 2001). Increasing the starting COD caused the accumulation of acetate in the medium, which can be explained by acetoclastic methanogenesis inhibition. The anaerobic oxidation of ethyleneglycol was negligible at 32 g/L starting COD, confirming that at this COD the activity of the granular sludge was completely inhibited.

Methanogenesis stimulation

Methane production values from the methanogenesis stimulation experiments were used to calculate the specific methane production rates for acetoclastic and hydrogenotrophic methanogenesis shown in Figure 7.2. As can be seen, the rate profiles of both processes differ considerably. A lag-time is observed in the acetoclastic experiments and the curves show a Gaussian-like profile with a maximum. In contrast, the maximum hydrogenotrophic methanogenesis rates occurred at the beginning of the experiments and a continuous quasi-linear decay takes place. A detailed study of the stimulation of methanogenesis was carried out using the results of Figure 7.2. Figure 7.3.a shows the overall methane production in both the acetoclastic and hydrogenotrophic methanogenesis experiments.

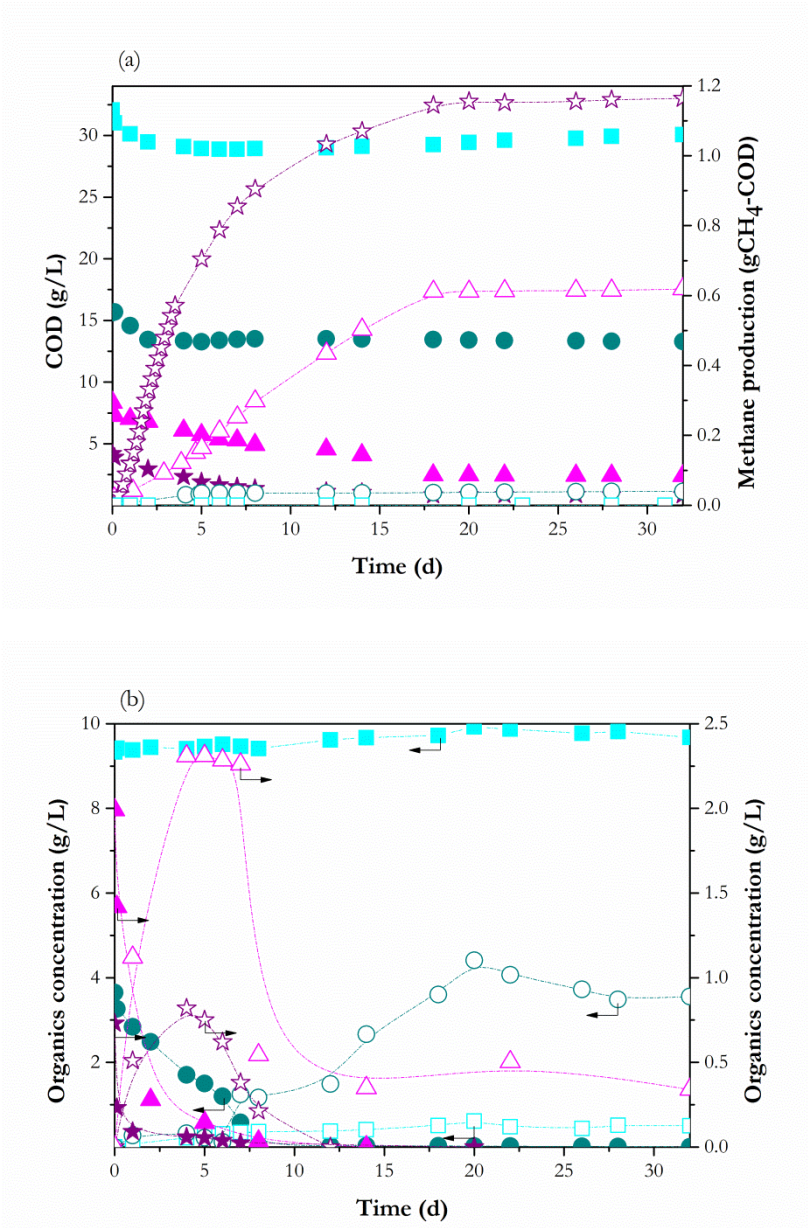

Figure 7.1. Evolution of COD (a), ethylene-glycol (b) (filled symbols), methane production (a) and acetate (b) (open symbols) during biodegradability assays at initial COD of 32 (squares), 16 (circles), 8 (triangles) and 4 (stars) g/L.

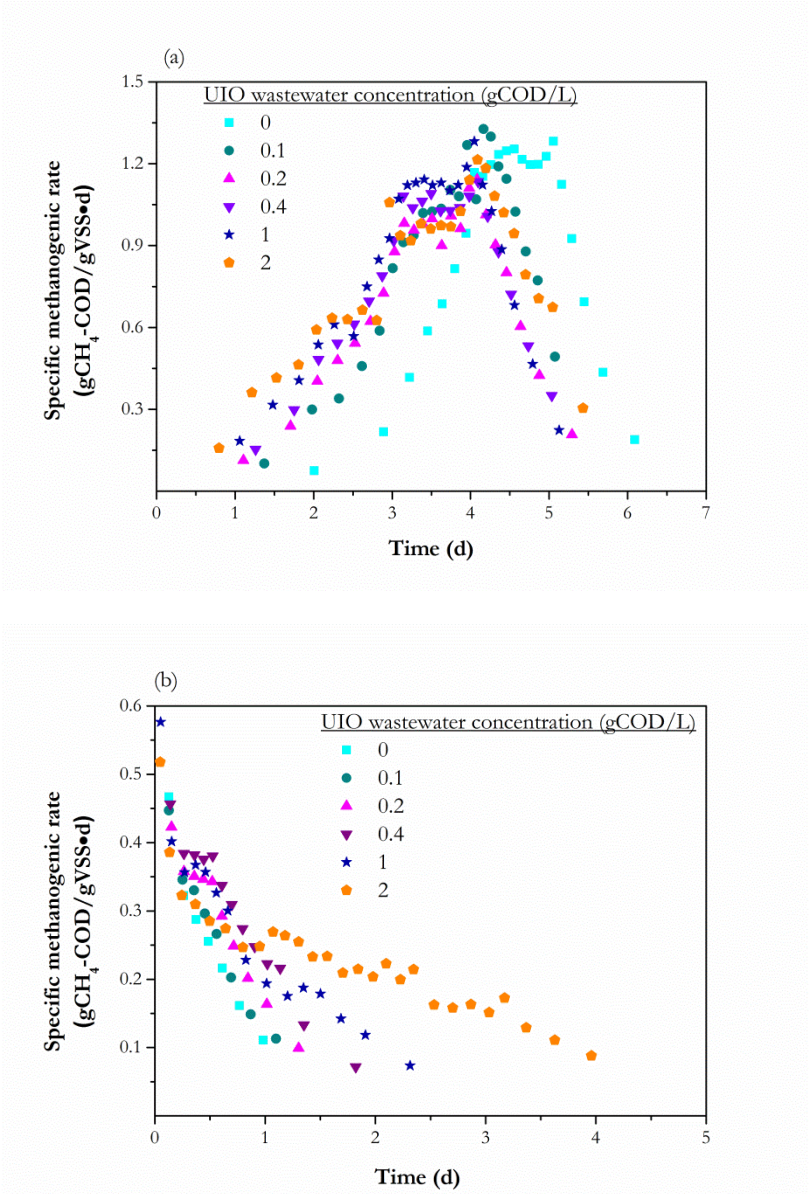

Figure 7.2. Time evolution of specific methanogenic rate from acetoclastic (a) and hydrogenotrophic (b) methanogenesis stimulation experiments with different UIO wastewater concentrations added.

The methane production obtained when adding UIO at 2 gCOD/L was 1.2 and 3 times higher than that obtained in the blank experiments with acetate and formiate, respectively. This fact indicates that hydrogenotrophic methanogenesis stimulation produces an improvement of the UIO wastewater methanization. Acetoclastic and hydrogenotrophic SMA values followed different patterns with increasing UIO wastewater COD (Figure 7.3.b). The enhancement of the SMA upon stimulation of the hydrogenotrophic methanogens was higher than that observed for the acetoclastic methanogens. Maximum values of 0.58 and 0.48 gCH_4-COD/gVS·d were obtained for UIO wastewater COD of 0.5 and 2 g/L, respectively. The complexity of the results from the acetoclastic experiments required a more detailed analysis. Maximum, average and initial specific acetoclastic rates were calculated and are depicted in Figure 7.4.a. The UIO wastewater COD did not affect significantly the maximum specific rates, thus non-competitive inhibition phenomena in the acetoclastic methanogenesis seem negligible at the organic loads tested. However, the initial and average specific methanogenic rates increased, which could be related with the occurrence of the initial lag phase. Nevertheless, increasing UIO wastewater COD shortened the time required to reach the maximum specific rate (Figure 7.4.b), which suggests that the UIO wastewater has a beneficial effect on the adaptation of the granular sludge to produce methane from acetate.

EGSB reactor performance

The performance of the EGSB during long-term operation is depicted in Figure 7.5. In order to avoid inhibition of the granular sludge, the OLR was gradually increased over 40 d at room temperature until reaching an OLR value of 10 gCOD/L·d. This operating strategy enabled COD removal efficiencies higher than 70 % and methanogenic potential of around 0.55 gCH_4/gCOD. Under these operating conditions, ethylene-glycol and the resulting acetate from its anaerobic oxidation were almost completely consumed. During the experiment, COD removal efficiencies suffered a slight reduction of 6 and 17 % when applying OLR of 2.5 and 5.5 gCOD/L·d, respectively.

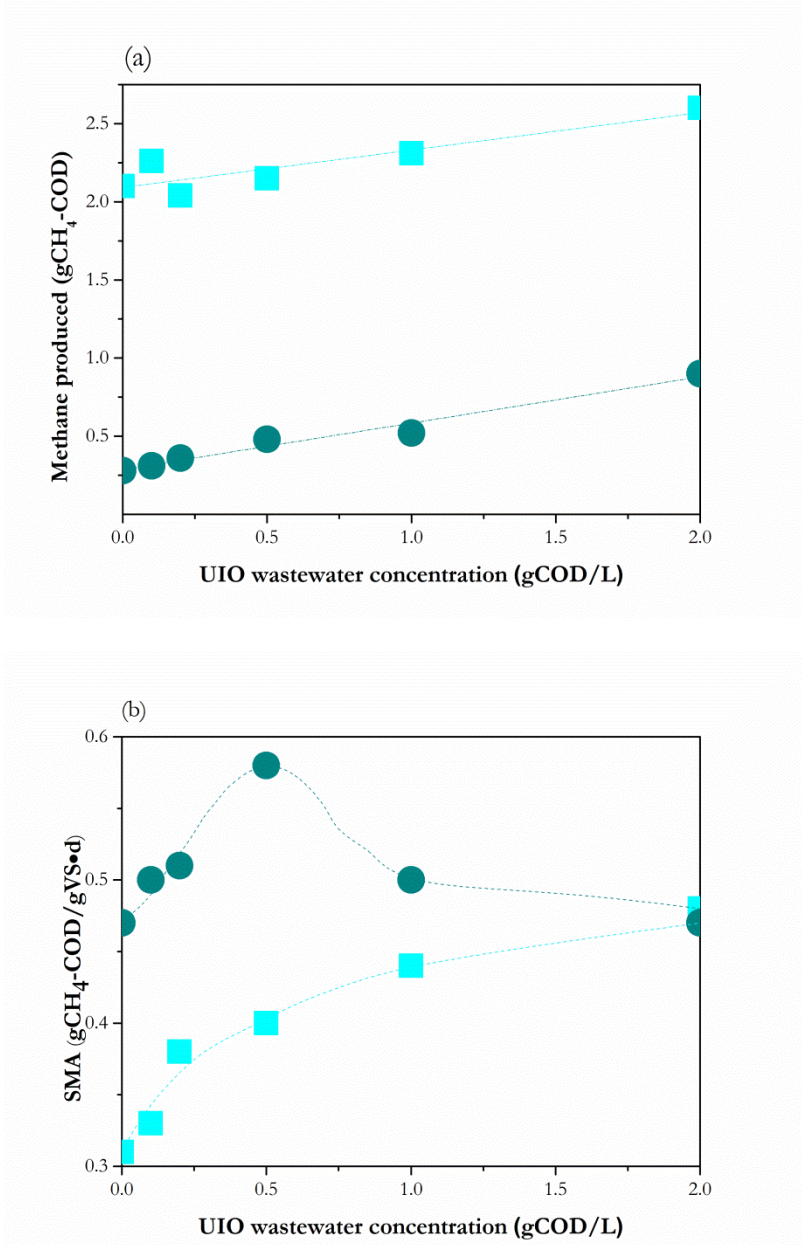

Figure 7.3. Comparison of the UIO-dependency for methane production (a) and SMA (b) in the acetoclastic (squares) and hydrogenotrophic (circles) methanogenesis stimulation experiments.

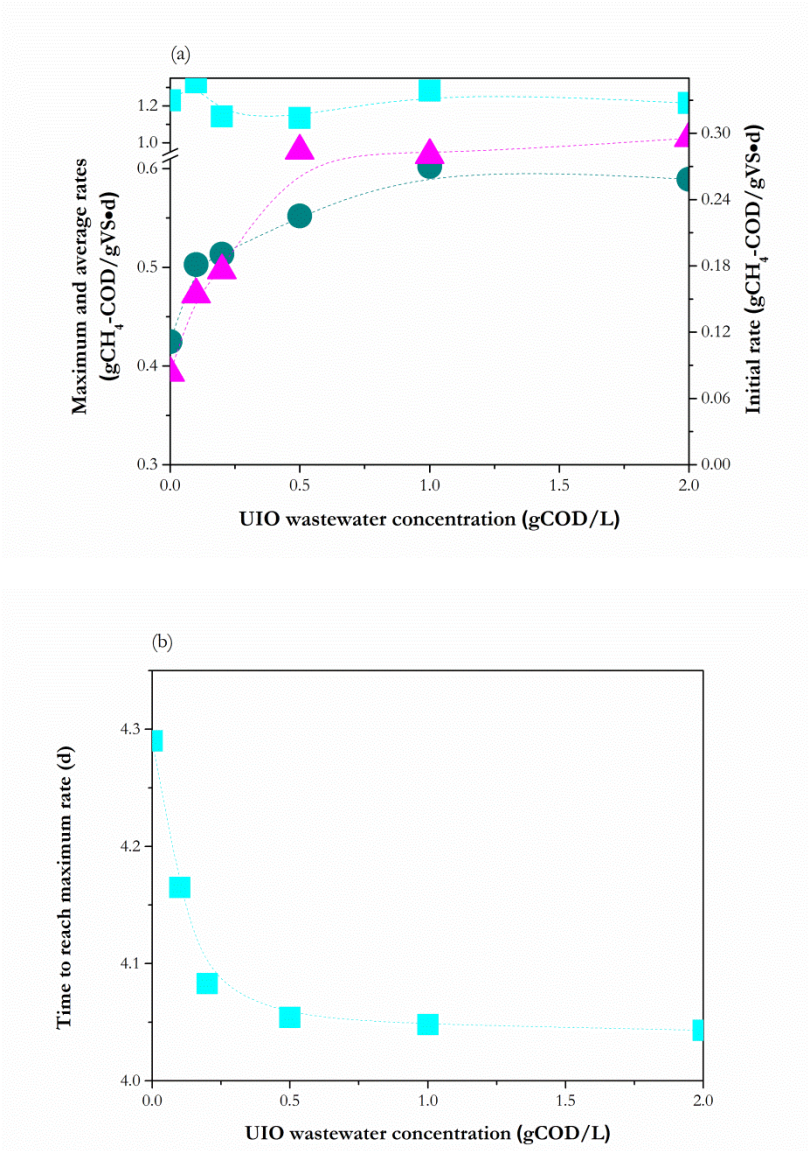

Figure 7.4. Maximum (squares), average (circles) and initial (triangles) rate (a) and time to reach maximum rate (b) for acetoclastic methanogenesis.

The increase of the OLR up to 10 gCOD/L·d caused a drop in COD removal and methanogenic efficiency. Meanwhile, acetate concentrations close to 3 g/L were detected in the resulting effluents. This indicates inefficient removal of the acetate generated from

anaerobic oxidation, which can be related with inhibition of the acetoclastic methanogenesis caused by increased toxicity and the low operating temperature. To recover reactor performance, the temperature was set at 32 ºC. A significant improvement in reactor performance was observed, resulting in COD removal and methanogenic efficiency of around 75 and 30 %, respectively, while the acetate concentration was lowered below 0.5 g/L. From these results it can be concluded that controlling the temperature within the mesophilic range enabled further acclimation of the biomass, which was necessary to maintain reactor performance.

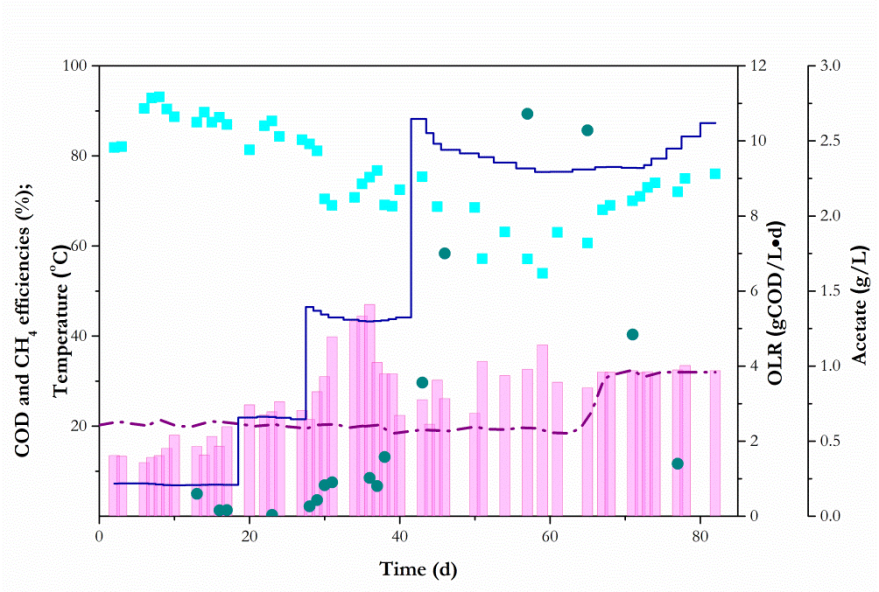

Figure 7.5. EGSB reactor performance. COD removal (squares) and methane production (bars) efficiencies, temperature (dash-dot line), OLR (solid line) and acetate concentration in the effluent (circles).

The composition of the UIO wastewater and the effluent from the EGSB reactor were analysed by GC/MS and HPLC/RI. Figure 7.6 depicts representative GC/MS chromatograms showing a fairly complex composition, summarized in Table 7.1. Most of the starting compounds were not detected in the resulting effluent after 80 d of

continuous operation (Figure 7.6.b). However, some intermediate compounds were detected in the effluent, such as phenolic hydrocarbons as well as trace concentrations of acetate, propionate and ethanol, indicating incomplete anaerobic oxidation. The partial inhibition of biogas production and the decrease in biodegradability of the UIO wastewater can be caused by the presence of substituted phenolic compounds (Olguin-Lora et al., 2003; Hernandez and Edyvean, 2008).

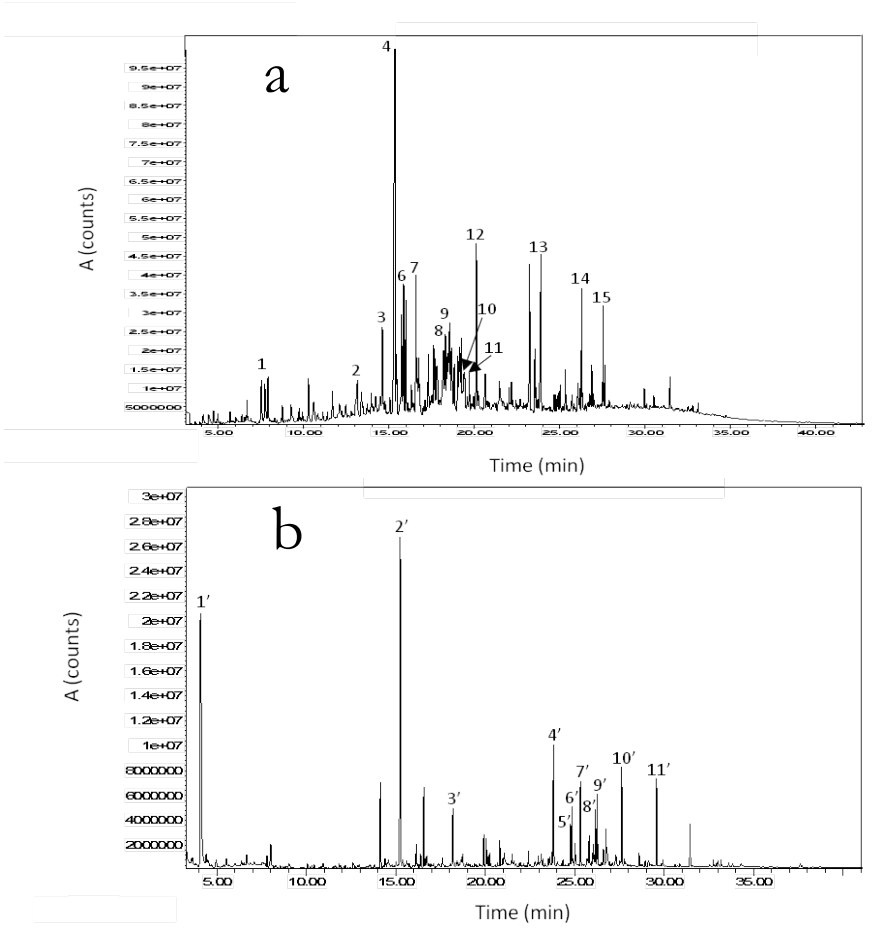

Figure 7.6. Charasteristic GC/MS chromatograms for EGSB influent (a) and effluent (b).

Evolution of the microbial population of the granular sludge

Figure 7.7 shows the DGGE band patterns for the archaea and bacteria domains from the anaerobic granular sludge at the startup and after 90 d of EGSB reactor continuous operation. Type and number of bacteria and archaea band patterns changed significantly during the experiment because of the specialization of the granular sludge to treat UIO wastewater. Bands were excised, reamplified and sequenced for microbial identification by means of the NCBI and RDP databases (Table 7.2). A clear reduction of the archaeal diversity in the granular sludge took place and most of the identified *Methanosaeta* sp. (A2, A3, A4 and A5) species disappeared. However, the identified A1–A8 *Methanosaeta* sp. prevailed, since this species has been reported to be competitive in wastewater contaminated with hydrocarbons (NCBI access number HQ689197). New species of archaea (A6 and A7) belonging to the *Thermoplasmatales* order appeared during the experiment, which have been identified in UASB reactors treating alkanes-bearing wastewater (Mbadinga et al., 2011) and usually appear in extremophilic environments (Brochier-Armanet et al., 2011).

With regard to the bacteria domain, a clear specialization of the anaerobic consortium occurred since only two DGGE bands remained (B3–B11 and B4–B12). These bands correspond to two species belonging to the phylum *Firmicutes*, being one of them related with the genus *Streptococcus* sp. (B3–B11) (Scheithauer et al., 2009), which have been found in waste digestion systems (Riviere et al., 2009; García et al., 2011). These species could be involved in the hydrolysis of the particulate matter of UIO wastewater. Owing to the adaptation of anaerobic granular sludge the rest of the identified bacteria suffered a clear modification. It is noteworthy that several species of nitrogen-consuming bacteria emerged, two of them belonging to the *Synergistaceae* family (B15, B16), and other to the species *Aminiphilus circumscriptus* (B17).

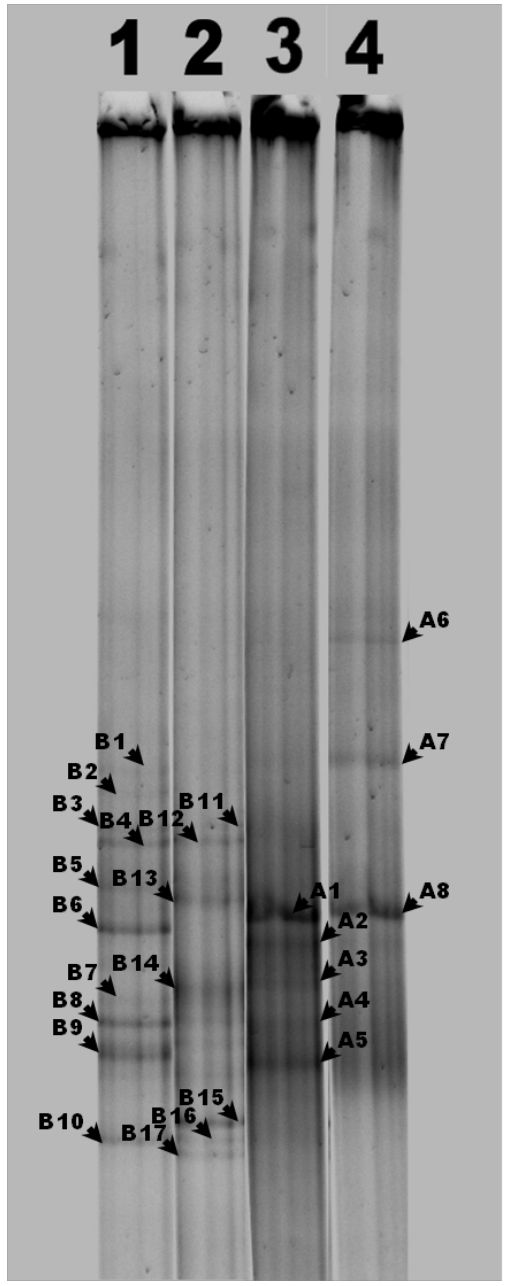

Figure 7.7. DGGE banding pattern of 16S rRNA amplified using universal primers for Bacteria (1, 2) and Archaea (3, 4) domains of the initial anaerobic granular sludge inoculum (1, 3) and upon the continuous experiment (2, 4).

Table 7.1. GC/MS characterization of the influent and effluent from EGSB reactor.

Influent		Effluent	
Number*	Compound	Number*	Compound
1	1-butanol	1'	methyl isobutyl ketone
2	tetradecane	2'	2-ethyl-1-hexanol
3	octyl-cyclopropane	3'	acetophenone
4	1-tetradecene	4'	2-methyl-phenol
5	undecane	5'	2-ethyl-phenol
6	3-methyl-2-pentene	6'	2,5-dimethyl-phenol
7	1-nonanol	7'	2-(1-methylethyl)-phenol
8	5-octadecene	8'	3,4-dimethyl-phenol
9	2-methyl-2-undecanethiol	9'	4-ethyl-phenol
10	1-bromo-pentadecane	10'	*p*-tert-butyl-phenol
11	1,4-dimethyl-cis-cyclooctane	11'	indole
12	3-tetradecene	HPLC/RI	Ethanol
13	Diphenyl ether	HPLC/RI	Acetate
14	2-(1,1-dimethylethyl)-phenol	HPLC/RI	Propionate
15	3,5-bis(1-methylethyl)-phenol		
HPLC/RI	Ethlylene-glycol		
HPLC/RI	Propylene-glycol		

* HPLC/RI: Compound identified by HPLC coupled to a refractive index detector.

Table 7.2. Identification of the DGGE bands amplified with specific primers for archaea (A) and bacteria (B) present in the granular sludge from the EGSB reactor.

DGGE band	Sequence with higher homology*	Similarity (%)	NCBI GenBank access number	RDP taxonomical hierarchy	Ref.**
A1	Uncultured *Methanosaeta* sp.	100 %	HQ689197	*Methanosaeta* sp. (100 %)	U
A2	Uncultured *Methanosaeta* sp.	95 %	JF754496.1	*Methanosaeta* sp. (95 %)	Wang et al., 2011
A3	Uncultured *Methanosaeta* sp.	97 %	JF754496.1	*Methanosaeta* sp. (97 %)	Wang et al., 2011
A4	Uncultured *Methanosaeta* sp.	96 %	HQ689197	*Methanosaeta* sp. (90 %)	U
A5	*Methanosaeta concilii*	99 %	CP002565.1	*Methanosaeta* sp. (100 %)	Barber et al., 2011
A6	Uncultured *Thermoplasmata* archaeon	99 %	JF754533.1	Archaea (100 %), *Thermoplasmatales* (76%)	Wang et al., 2011
A7	Uncultured *Thermoplasmata* archaeon	96 %	JF754533.1	Archaea (100 %), *Thermoplasmatales* (50%)	Wang et al., 2011

A8	Uncultured *Methanosaeta* sp.	93 %	HQ689197	Archaea (100 %), *Methanosaeta* sp. (80 %)	U
B1	Uncultured *Bacterioidetes* bacterium	90 %	HQ183944.1	Bacteria (92 %), *Planctomycetes* (16 %)	Liu et al., 2011
B2	NS			Bacteria (96 %), Crysiogenetes (14 %)	-
B3	uncultured *Streptococcus* sp.	96 %	EU704223.1	Bacteria (100 %), Firmicutes (54 %)	Scheithauer et al., 2009
B4	uncultured Firmicutes bacterium	95 %	CU918169.1	Bacteria (100 %), Firmicutes (47 %)	Riviere et al., 2009
B5	Uncultured bacterium	96 %	GU325923.1	Bacteria (100 %), Firmicutes (38 %)	U
B6	uncultured *Syntrophobacter* sp.	97 %	EU888828.1	*Syntrophobacter* sp. (97 %)	Worm et al., 2009
B7	*Trichococcus flocculiformis*	98 %	NR_042060.1	*Trichococcus* sp. (100 %)	Liu et al., 2002
B8	NS			Bacteria (96 %), Bacteroidetes (19 %)	-
B9	Uncultured bacterium	95 %	AB470353.1	Bacteria(99 %), Proteobacteria (75 %), Gammaproteobacteria (37 %)	Iguchi et al., 2009

B10	Uncultured bacterium	95 %	AB470353.1	Bacteria(99 %), Proteobacteria (63 %), Gammaproteobacteria (40 %)	Iguchi et al., 2009
B11	NS			Bacteria (100 %), firmicutes (37 %)	-
B12	uncultured bacterium	92 %	JF595815	Bacteria (100 %), firmicutes (57 %)	García et al., 2011
B13	uncultured Chloroflexi bacterium	100 %	CU917962	Bacteria (100 %), chloroflexi (33 %)	Riviere et al., 2009
B14	*Desulfomicrobium* sp.	99 %	AY570692	*Desulfomicrobium* sp. (100 %)	Grabowski et al., 2005
B15	Uncultured Aminanaerobia bacterium	97 %	CU917491	Bacteria (100 %), Synergistaceae (53 %)	Riviere et al., 2009
B16	Uncultured bacterium	93 %	HQ453309	Bacteria (100 %), Synergistaceae (22 %)	Zhang et al., 2011
B17	*Aminiphilus circumscriptus*	99 %	NR_043061	*Aminiphilus* sp. (100 %)	Díaz et al., 2007

*NS=BLAST tool returns Non Significant responses for any query length; *U=Unpublished

7.4. CONCLUSIONS

UIO wastewater can be efficiently biotreated by anaerobic granular sludge in an EGSB reactor, where the specialization of the biomass leads to significant changes in the microbial composition of the granular sludge. At low concentrations, this wastewater even enhances the specific methanogenic activity of both acetate- and hydrogen-consuming methanogens, allowing high methanogenic potential. Anaerobic biodegradation at room temperature is feasible at moderate OLR values, but it is necessary to increase the temperature for OLRs higher than 5.5 gCOD/L·d. The observed inhibition of anaerobic microbial activity can be caused by the presence of some identified inhibitory compounds such as substituted phenols.

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CONCLUSIONES

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Los resultados obtenidos en la presente Tesis Doctoral han permitido establecer las siguientes conclusiones para cada uno de los estudios realizados:

Biodegradacion de inhibidores de corrosión en condiciones anerobias

1. Los inhibidores de corrosión con 1 ó 2 átomos de nitrógeno (quinoleína, morfolina y piperazina) pueden ser degradados en condiciones anaerobias, mientras que el benzotriazol constituido por 3 átomos de nitrógeno es refractario a este tratamiento.
2. La biodegradación de quinoleína se vio severamente afectada por la presencia de otros inhibidores de corrosión, disminuyendo drásticamente su velocidad inicial de degradación. Sin embargo, la degradación de piperazina mejoró significativamente en presencia del resto de compuestos heterocíclicos estudiados. En el caso de morfolina y benzotriazol no se observaron mejoras significativas en la degradación de ambos compuestos en presencia de los otros inhibidores analizados.
3. Las arqueas acetoclásticas resultaron inhibidas irreversiblemente por la presencia de quinoleína. No obstante, no se observó ningún efecto inhibitorio sobre la metanogénesis hidrogenotrófica en presencia de los inhibidores de corrosión estudiados.
4. Mediante tecnología EGSB pudieron tratarse aguas sintéticas conteniendo los inhibidores de corrosión estudiados. En el caso de quinoleína y piperazina se trataron cargas de 71 y 98 mg/L·d, respectivamente, manteniendo una elevada

eliminación de DQO (80 %). Sin embargo, la producción de metano si se vio severamente afectada, reduciendo su rendimiento inicial a la mitad (0,38 gCH_4-DQO/gDQO).

Eliminación biológica anaerobia de pesticidas comerciales

5. De los compuestos fitosanitarios estudiados, el MCPA presenta una baja biodegradabilidad anaerobia, mientras que el imidacloprid y el dimetoato necesitan al menos 40 d para ser degradados completamente. A partir de los intermedios de degradación detectados (nitrosoguanidina, metabolito desnitro/guanidina y metabolito urea), el imidacloprid se biodegrada vía reducción del grupo nitro. El dimetoato presenta dos posibles rutas de degradación, una a través del ataque al grupo alcoxi, ya que se identificó el derivado desmetilo del dimetoato, y la otra mediante la eliminación del grupo metilo unido a la amina al detectarse como intermedio el derivado tioato del dimetoato.

6. Las mezclas binarias y ternarias de los pesticidas objeto de estudio ejercen un efecto antagónico sobre la degradación de MCPA y dimetoato, no mejorándose la degradación de MCPA y disminuyendo a la mitad la de dimetoato. En el caso de imidacloprid, la presencia del resto de pesticidas estudiados aumenta significativamente su velocidad inicial de degradación.

7. Los insecticidas (imidacloprid y dimetoato) provocan una inhibición irreversible sobre las arqueas acetoclásticas, reduciendo su actividad un 92 %, mientras que únicamente el dimetoato es tóxico para las arqueas hidrogenotróficas, poniendo de manifiesto que éstas son más resistentes que las acetoclásticas. Esta afirmación se corrobora mediante el análisis de DGGE, observándose que las arqueas hidrogenotróficas pertenecientes al género *Methanobacterium* predominan en el lodo granular durante el tratamiento en continuo.

8. El empleo de reactores anaerobios de alta eficacia, tipo EGSB, suponen una alternativa viable para la tratamiento de aguas que contienen pesticidas, alcanzándose eficiencias superiores al 85 %, aplicando cargas de 87, 29 y 38 mg/L de MCPA, imidacloprid y dimetoato, respectivamente.

Viabilidad del tratamiento anaerobio de un agua residual procedente del lavado de tanques de fabricación de productos fitosanitarios

9. El tratamiento biológico anaerobio de un agua residual industrial que contiene productos fitosanitarios utilizando un reactor de alta eficacia (EGSB) permite, tanto en condiciones mesofílicas (35 ºC) como termofílicas (55 ºC), la eliminación del 97 % de los pesticidas que contienen dichas aguas, sin que la producción de metano se vea significativamente afectada.

10. Las arqueas hidrogenotróficas presentan una mayor resistencia a la presencia de pesticidas que las acetoclásticas, las cuales son prácticamente inhibidas incluso para una concentración de 1,8 gDQO/L. En el caso de las hidrogenotróficas reducen su actividad a la mitad con una concentración de 12,8 gDQO/L. Este hecho se confirma durante la operación de tratamiento en continuo de estas aguas residuales, observándose que existe un metabolismo eficiente de propionato y butirato, lo que solo se consigue cuando la concentración de H_2 es baja debido a la acción de las arqueas hidrogenotróficas.

Tratamiento biológico de un agua residual industrial procedente del reciclado de aceites industriales usados mediante un reactor anaerobio de alta eficacia

11. La biodegradación anaerobia de este tipo de aguas a temperatura ambiente (17-21 ºC) empleando un reactor de alta eficacia (EGSB) permite tratar velocidades de carga orgánica

hasta 5,5 gDQO/L·d. Sin embargo, a temperaturas en el rango mesofílico (32 ºC) es posible alcanzar velocidades de carga orgánica de 10,5 gDQO/L·d manteniendo el rendimiento metanogénico.

12. El empleo de cargas por debajo de 2 gDQO/L favorece tanto la actividad metanogénica acetoclástica como la hidrogenotrófica, aumentando hasta 3 veces la producción de metano respecto a un ensayo en blanco que emplea como única fuente de carbono una mezcla de acetato/formiato.

CONCLUSIONS

The results presented in this work support the following conclusions:

Biodegradation of corrosion inhibitors under anaerobic conditions

1. Corrosion inhibitors with 1 or 2 nitrogen atoms (quinoline, morpholine and piperazine) can be biodegraded under anaerobic conditions while benzotriazole, which contains 3 nitrogen atoms were not removed.

2. Quinoline degradation was severely affected by the presence of other corrosion inhibitors, decreasing drastically its initial removal rate. However, piperazine degradation improved significantly in presence of the other N-heterocyclic compounds studied. Morpholine and benzotriazole degradation was not showed a significant improvement in presence of the other corrosion inhibitors.

3. Acetoclastic archaea were irreversible inhibited by the presence of quinoline. However, no inhibitory effect over hydrogenotrophic methanogenesis in presence of other corrosion inhibitors was observed.

4. Anaerobic treatment (EGSB) achieved quinoline and piperazine removal efficiencies above 97 % when treating a feed with quinoline and piperazine loads of 71 and 98 mg/L·d, respectively, maintaining a high COD removal efficiency. However, methane production decreased by 50 % at increasing targets concentration, reaching a methane production of 0.38 gCH_4-COD/gCOD consumed.

Anaerobic biological degradation of commercial pesticides

5. Imidacloprid and dimethoate showed certain biodegradability after an acclimation period of 40 d. However, MCPA was

poorly biodegraded. According to the intermediates detected (nitrosoguanidine, desnitro/guanidine metabolite and urea metabolite) imidacloprid biodegradation occurred through the reduction of nitro group. Dimethoate biodegradation followed two possible pathways, the attack of the alkoxy group, since des-methyl was detected, or the demethylation of the methylamine moiety producing thioate.

6. Binary and tertiary mixtures caused an antagonistic effect over MCPA and dimethoate degradation efficiency, MCPA biodegradation was not improved and dimethoate degradation was reduced by a 50 %. However, imidacloprid initial removal rate was enhanced by the presence of the other pesticides studied.

7. Insecticides provoked an irreversible inhibition over the acetoclastic archaeas decreasing their activity by a 92 %, while dimethoate was only toxic for hydrogenotrophic biomass. This fact corroborates that hydrogenotrophic archaeas are more robust against toxic shocks than the acetoclastic ones. DGGE analysis showed that *Methanobacterium* genus, a hydrogenotrophic archaea, prevailed in the granular biomass during the long-term experiment.

8. High-rate anaerobic reactors offer a feasible alternative for treating pesticides bearing wastewater, achieving pesticides removal efficiencies above 85 % when treating pesticides loading rates of 87, 29 and 38 mg/L·d of MCPA, imidacloprid and dimethoate, respectively.

Anaerobic treatment of wastewater from a pesticides factory

9. Industrial wastewater containing pesticides can be effectively treated in a high-rate (EGSB) reactor at mesophilic (35 ºC) and thermophilic (55 ºC) conditions, reaching a pesticide removal efficiency of 97 % without affecting methane production.

10. Hydrogenotrophic archaeas are more resistant towards pesticides than the acetoclastic biomass which was completely inhibited even at low COD concentrations (1.8 gCOD/L). Activity of hydrogenotrophic archaeas suffered a decrease of 50 % when treating wastewater with a COD concentration of 12.8 gCOD/L. This fact is confirmed with the results achieved in the continuous experiment where a efficient metabolism of propionate and butyrate took place due to the low hydrogen concentration in the reaction medium because of the hydrogenotrophic biomass activity.

Anaerobic treatment of wastewater from used industrial oil recovery in a high-rate reactor

11. This wastewater can be effectively treated by anaerobic biomass at room temperature (17–21 ºC) and applying organic loading rates (OLR) lower than 5.5 gCOD/L·d. A further increase of the OLR up to 10 gCOD/L·d cause a detrimental effect on the system performance, making necessary a temperature control at mesophilic conditions (32 ºC).

12. Low-strength wastewater with a COD concentration lower than 2 g/L improved the specific activity of the acetoclastic and the hydrogenotrophic methanogens, increasing up to 3 times the methane production than that obtained in the blank experiments with acetate/formiate as sole carbon source.